MESSENGER RNAs AND PROTEIN SYNTHESIS

IN

DEVELOPING CHLOROPLASTS

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DECLARATION

All the work reported in this thesis was carried out by myself with the exception of the preparation of certain enzymes and other materials listed in Chapter 2 (Section 2.1.2.) and elsewhere in the text. No part of the work described has been reported elsewhere for the award of any other degree or diploma

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ABSTRACT

Measurements were made of some of the changes that occur in plastid protein synthesis during the greening of dark grown pea seedlings. Several parameters of protein synthesis were studied to determine the nature of the controls responsible for the observed patterns of protein synthesis and accumulation. It was found that transcriptional control was responsible for neither the quantitative nor the qualitative changes in protein synthesis. The regulation of protein synthesis appeared to be due to changes in the efficiency with which the various plastid mRNA species acted as templates for translation. The large subunit of ribulose-bisphosphate carboxylase (LSU) was shown to have its synthesis differentially stimulated at various periods during chloroplast formation due to variations in the ability of the LSU mRNA to compete for plastid ribosomes and to initiate protein synthesis. Some evidence is also presented to suggest that the LSU polypeptide in peas and spinach is synthesised via a precursor molecule slightly larger than the final protein product.

The general conclusion reached is that the specificity of protein syntheisis in developing pea plastids is due to translational control, probably acting at the initiation of protein synthesis.

Certain properties of spinach chloroplast messenger RNAs were examined to gain information on the coding functions of the plastid genome and the possible involvement of mRNA structure in regulating protein synthesis. A range of RNA fractionation procedures was used to attempt to purify, and to identify,

specific chloroplast mRNA species. Ten size classes of messenger RNAs were identified and four of these were mapped. All mapped to discrete regions of the plastid DNA molecule suggesting that the messenger RNAs present in spinach chloroplasts are, generally, the products of transcription of the plastid DNA. The possible involvement of mRNA stability and conformation in the regulation of protein synthesis is also discussed.

CHAPTER 1

Introduction

It is only about ten to fifteen years since the semi-autonomy of certain organelles has been clearly recognised. Now it is evident that, in addition to the DNA of the nucleus, nearly all eukaryotes possess DNA in chloroplasts or in mitochondria or both, in flagellar bases and possibly in centrioles. Although the function of these organelles is clear, their presence raises questions of their origin, their degree of autonomy and their interaction with nuclear-coded proteins and activities. The chloroplasts of flowering plants are easily separated from most nuclear and cytoplasmic material and many of the characteristic reactions of chloroplasts can be carried out in the isolated organelles *in vitro*. For these reasons there is already a large amount of information regarding the photosynthetic pathways and their relation to chloroplast structure and the mechanisms of chloroplast gene expression. However, there is still little information available on the interrelationships between gene expression of mature photosynthesising chloroplasts and their organellar precursors, the proplastids and etioplasts. In particular, there are few data on the nature of any control systems that may exist and the degree of nuclear intervention in development.

The assumption is frequently made that chloroplast development represents a unidirectional process, with small, colourless proplastids giving rise to differentiated types of plastids such as chloroplasts, amyloplasts, chromoplasts and etioplasts (Kirk and Tilney-Bassett, 1978). Proplastids represent a part of the normal cell life cycle only in the more highly evolved algae and land plants (Pickett-Heaps, 1975). With few exceptions, the photosynthetic lamellae of more primitive organisms are always retained either in the form of thylakoids free in the cytoplasm (in the prokaryotes) or envelope-bound in chloroplasts (in the lower eukaryotes). However, the suggestion has been made that the mature chloroplast in higher algae and plants exists in a state of equilibrium with the proplastid (Whatley, 1978). If thylakoid renewal falls behind thylakoid degradation, the chloroplasts may de-differentiate into proplastids; in the reverse case, that of greater rate of thylakoid synthesis than degradation, the proplastid will form the chloroplast in the light or the etioplast in the dark. Similarly the transition of etioplast to mature chloroplast in response to light may represent a further shift in the equilibrium of thylakoid production and degradation.

Most available information about the differentiation of the chloroplast has come from studies of the etioplast to chloroplast transformation when dark grown plants are transferred to light. In some respects the classical, fully-formed etioplast with its large crystalline prolamellar body is an aberrant system. The conditions of long dark growth necessary for its formation would rarely occur in nature (Kirk and Tilney-Bassett, 1978). However, the

assumption is made that the biosynthetic processes and control mechanisms that operate during chloroplast formation from etioplasts are the same as for natural chloroplast development under regular, alternating day and night conditions. The greening of etiolated seedlings, therefore, provides a useful and convenient experimental system.

In angiosperms the etioplast represents the furthest the plant can proceed towards chloroplast formation in the absence of light. Consequently the etioplast contains many of the components necessary for a functional chloroplast enabling the rapid transformation of the etioplast to a photosynthetically active body. Photosystem I comes into operation (at low rates) only minutes after transfer of dark grown plants to light and Photosystem II activity first appears after thirty minutes or longer (Henningsen and Boardman, 1973). However, despite these rapid responses to light, high rates of synthesis of chloroplast components are still necessary for the complete transformation to occur. It is, therefore, not surprising that amongst a wide range of biochemical components of photosynthesis, proteins show a large increase in amount or activity when dark grown plants are placed in the light. Some of this new synthesis will occur in the cytoplasm of the cell but some will also result from the synthetic activity of the etioplast. The bipartite nature of the synthesis of plastid proteins poses problems in determining the origins of changes observed in chloroplast development. A paucity of knowledge of the function of the chloroplast chromosome and the mechanism of its expression compounds the problem.

1.1 *Gene Expression in Plastids*

Chloroplasts contain all the components necessary for protein synthesis including ribosomes, tRNAs, aminoacyl-tRNA synthetases, initiation and elongation factors, messenger RNAs, RNA polymerase and DNA. The chloroplast DNA is a circular molecule of 83-95 million daltons (contour length of 40-46 μm) in higher plants (Herrmann *et al.*, 1975) and 75-128 million daltons (36-62 μm in contour) in algae (Kirk and Tilney-Bassett, 1978). In pea plastids there may be from 14 to 59 copies of this DNA per plastid (Bennett and Radcliffe, 1975, Kowallik and Herrmann, 1972) although over one hundred copies per plastid have been reported in *Beta vulgaris* (Herrmann *et al.*, 1974).

A DNA molecule as large as that of the chloroplast has the potential to code for the plastid-specific ribosomal and transfer RNA's and about 100 proteins of 40,000 daltons. The ribosomal genes have been mapped in the DNA of several plants. There are two copies of the ribosomal genes per DNA molecule in *Zea mays* (Bedbrook *et al.*, 1977), spinach (Whitfeld *et al.*, 1978) and *Chlamydomonas* (Rochaix, 1978, Rochaix and Malnoe, 1978) on an inverted repeat region of the DNA (Kolodner and Tewari, 1979). The ribosomal genes of *Euglena gracilis* plastids are located in three tandem repeat regions of about 4.6 kilobase pairs each, (Jenni and Stutz, 1978; Rawson *et al.*, 1978; Knopf and Stutz, 1978).

Haff and Bogorad (1976) estimated that 0.60 to 0.75 percent of the maize chloroplast DNA coded for transfer RNAs. This would be sufficient for between 20 and 26 tRNA cistrons. In spinach over 21

genes for transfer RNAs have been mapped in the plastid genome. Fifteen of these genes are located in the large single-copy region between the inverted repeats and three in the inverted repeats (Driesel *et al.*, 1979). Chloroplast genes have also been demonstrated to code for most, if not all, of the tRNA species in the plastids of tobacco (Tewari and Wildman, 1970), bean (Steinmetz and Weil, 1976), pea (Tewari *et al.*, 1977) and of the algae *Euglena* and *Chlamydomonas* (Hallick *et al.*, 1978; Malnoe and Rochaix, 1978).

Given that the chloroplast DNA codes for the ribosomal RNA's and the transfer RNAs, the problem still remains to determine what other coding functions this DNA carries. Only two proteins have been conclusively shown to be coded for by the plastid DNA; the large subunit (LSU) of ribulose-bisphosphate carboxylase (RuBPCase) (Coen *et al.*, 1977, Rochaix and Malnoe, 1978) and a 32,000 dalton membrane protein (Bedbrook *et al.*, 1978). RuBPCase is an oligomer of 16 subunits; 8 large, catalytic subunits (of about 55,000 daltons) and 8 small subunits (of about 14,000 daltons) of unknown function (for a review on structure, function and synthesis of RuBPCase and its subunits see Bottomley, in press). LSU is the major soluble product of protein synthesis by isolated chloroplasts of pea (Blair and Ellis, 1973), spinach (Bottomley *et al.*, 1974) and *Euglena* (Vasconcelos, 1976). Evidence that the LSU gene is located on the chloroplast genome has come from genetic studies (Chan and Wildman, 1972; Sakano *et al.*, 1974), from the translation of chloroplast mRNA to yield LSU (Hartley *et al.*, 1975) and from the transcription and translation of chloroplast DNA to yield the LSU polypeptide.

Bottomley and Whitfeld (1979) identified the large subunit by partial proteolytic digestion as one of the products of an *E. coli* cell-free transcription-translation system directed by spinach chloroplast DNA. A fragment of maize chloroplast DNA carrying the gene for the LSU protein has been cloned in a bacterial plasmid (Coen *et al.*, 1977; Bedbrook *et al.*, 1979) and its location on a restriction endonuclease map established. The LSU gene has also been mapped in *Chlamydomonas* (Rochaix and Malnoe, 1978).

Information about the types of messenger RNA present in chloroplasts derives primarily from the examination of proteins synthesised by isolated chloroplasts. Protein synthesis by isolated plastids is not dependent upon the presence of DNA and is assumed to give proteins representative of the messenger population in the chloroplast (Whitfeld, 1977). Similarly, inhibitors such as lincomycin or chloramphenicol which are specific for the 70S chloroplast ribosomes or those for the 80S cytoplasmic ribosomes have been used to allow the synthesis of protein from only one source, plastid or cytoplasm. In this way a number of proteins have been tentatively identified as products of chloroplast protein synthesis (Ellis *et al.*, 1978).

Mendiola-Morgenthaler *et al.*, (1976) suggest that three of the five coupling factor subunits are made in chloroplasts. These subunits are those with molecular weights of 59,000 and 56,000 daltons and, probably also the 15,000 dalton subunit. Other likely products of chloroplast protein synthesis are: the chlorophyll-protein complex I protein (Hachtel, 1976); one or more of the ribosomal proteins (Yurina *et al.*, 1978), the chloroplast elongation factors EF-Tu and EF-G (Tiboni *et al.*, 1978), a protein involved in the stacking of thylakoid membranes into grana

and a number of other chloroplast membrane proteins (Machold and Aurich, 1972; Apel and Schweiger, 1973; Joy and Ellis, 1975), some of the cytochromes (Gregory and Bradbeer, 1973) and ACP-dependent fatty acid synthetase (Ernst-Farberg *et al.*, 1974). The last two categories may only be made by certain algal chloroplasts. Of the chloroplast membrane proteins, Machold and Aurich (1972) concluded that "the largest number of lamellar proteins is synthesised on the 70S ribosomes of chloroplast". Bottomley *et al.* (1974) suggested that as many as 15 membrane polypeptides were made by isolated spinach chloroplasts compared to five labelled by isolated pea and *Vicia* chloroplasts (Eaglesham and Ellis, 1974; Hachtel, 1976).

Although, as mentioned earlier, chloroplast DNA could potentially code for about 100 proteins each of 40,000 daltons, the entire genome does not appear to be transcribed at the one time, nor parts of it at similar rates. In *Euglena gracilis*, for example, only 23 to 26 percent of the chloroplast DNA is transcribed in the mature chloroplast (Chelm *et al.*, 1978; Chelm and Hallick, 1976). The transcripts were present in two frequency classes; high frequency-low complexity transcripts of about 10^6 copies per cell presumed to represent the ribosomal RNAs; and low frequency-high complexity transcripts of about 1.6 to 3.6 x 10^3 copies per cell. The low frequency class may represent the messenger RNA population.

Further evidence for the differential expression of chloroplast DNA has come from maize leaf cells. Link *et al.*, (1978) found that the gene for the LSU is transcribed only in the bundle sheath cells No translatable LSU messenger RNA was detected in the mesophyll cells, where the C_4 photosynthetic pathway occurs, even though regions on either side of the LSU gene appeared to be transcribed.

1.2 *Chloroplast Messenger RNAs and their Translation* in vitro

Some doubt still remains regarding the extent of polyadenylation of chloroplast messenger RNA. Wheeler and Hartley (1975) found that spinach chloroplast mRNA lacks poly-A, although tracts of less than 20 adenylate residues would not have been detected. These findings have been supported by Edelman *et al.*, (1977) and Howell *et al.*, (1977). Similarly the messenger RNA for the LSU does not contain poly-A (Wheeler and Hartley, 1975; Sagher *et al.*, 1976; Howell *et al.*, 1977). However, Haff and Bogorad (1976) isolated poly-A-containing RNA from maize seedlings and estimated that six percent of this poly-A-RNA hybridised to chloroplast DNA. Poly-A-containing RNA isolated from maize chloroplasts gave over 65 percent hybridisation to chloroplast DNA. These poly-A tracts were about 45 nucleotides long. More recently, Bartoff and Price (1979) found that poly-A-containing RNA was synthesised by isolated spinach chloroplasts. The poly-A tracts were from 10 to 45 residues long. The identification of a poly-A polymerase in wheat chloroplasts (Burkard and Keller, 1974) lends support to the conclusion that some chloroplast messengers are polyadenylated. There have been no reports of the polyadenylation of the messenger RNA for LSU.

The sizes given for chloroplast poly-A tracts closely resemble those published for mitochondrial mRNAs. This places the organellar messengers somewhere in between the bacterial mRNAs that lack poly-A and the eukaryotic messengers that frequently have a very long poly-A tract. The chloroplast ribosomes also appear to have many properties that are intermediate between the eukaryotic and prokaryotic ribosomes. These properties are summarised in Table 1.1.

Property	Source of Ribosomes		
	Cytoplasm	Chloroplast	Bacteria
Buoyant density (g/cm^3)	1.549-1.557	1.568-1.576	1.612-1.640
Protein content (%)	49.0-50.2	45.7-47.0	35.0-39.7
RNA/protein ratio	0.99-1.04	1.13-1.19	1.52-1.86
Molecular weight (daltons)	4.0×10^6	$3.0-3.1 \times 10^6$	2.5×10^6
Size (Å)	260x190-200	220x170	200x150
Sedimentation velocity (S)	80-87	67-70	70

Table 1.1 Properties of Chloroplast, Cytoplasmic and Bacterial Ribosomes. (Data from Oparin *et al.*, 1975; Smillie and Scott, 1969; Watson, 1965).

Despite the similarities and differences between chloroplast, cytoplasmic and bacterial ribosomes, the chloroplasts resemble bacteria in most aspects of protein synthesis. For example, in all known cases chloroplast transfer RNAs and aminoacyl tRNA synthetases differ from those of the cytoplasm but are frequently interchangeable with those of *Escherichia coli* (e.g, Guillemaut and Weil, 1975). Similarly, the *E. coli* and chloroplast initiation and elongation factors seem to be interchangeable (Sala *et al.*, 1970; Tiboni *et al.*, 1976). Consequently, chloroplast messenger RNAs from higher plants have been successfully translated in *E. coli* cell-free translation systems (Hartley *et al.*, 1975; Bottomley *et al.*, 1976). However, *Euglena* chloroplast mRNA has been translated in a wheat germ cytoplasmic translation system (Sagher *et al.*, 1976). The discrimination of various *in vitro* translation systems for messenger RNAs is discussed by Herrlich and Schweiger (1978) and Revel *et al.*, (1976).

1.3 *Co-ordination of Chloroplast and Cytoplasmic Protein Synthesis*

There is a strong dependence of chloroplasts on cytoplasmic protein synthesis. Conversely, chloroplast protein synthesis is important to the normal development of the cell. Hanson and Bogorad (1978) suggest "that chloroplast protein synthesis is essential for cellular growth even when photosynthesis is not essential".

The dependence of chloroplasts on protein synthesis in the cytoplasm can be seen most clearly in the extensive role of the cytoplasm in the production of chloroplast proteins. A list of the chloroplast proteins known to be made on the 80S cytoplasmic ribosomes and proteins containing subunits from both synthetic systems is given by Kirk and Tilney-Bassett (1978).

One such protein, the small subunit (SSU) of RuBPCase is of particular interest. There has been some dispute as to whether or not the synthesis of the small and large subunit proteins is under special coordinated control. The absence of any clear evidence demonstrating the existence of a pool of either subunit has added support to the notion of coordinated synthesis. Ellis *et al.*, (1978) suggested that nuclear-coded polypeptides control protein synthesis in chloroplasts and that LSU synthesis is subject to regulation by SSU. This is supported by studies on *Chlamydomonas* (Iwanij, 1975) and greening pea shoots (Ellis, 1975) using differential inhibitors of protein synthesis. In both organisms, inhibition of small subunit synthesis blocks ribulose-bisphosphate carboxylase synthesis but accumulation of LSU does not result. However, if LSU synthesis is blocked, as in mutant rye leaves, SSU will accumulate. This result was obtained by Feierabend and

Wildner (1978) using a temperature-induced chloroplast ribosome deficiency. At 30°C these plants were inhibited in chloroplast protein synthesis and, consequently produced no LSU. On the other hand, RuBPCase deficient mutants of *Oenothera* (Hallier *et al.*, 1978) and *Chlamydomonas* (Givan, 1979) gave no SSU synthesis even though the mutation was shown to be localised in the plastid DNA. Such a result implies that large subunit synthesis affects SSU synthesis. Yet another conclusion was reached by Barraclough and Ellis (1979). In isolated soybean leaf cells, the single blockage of either SSU or LSU synthesis with inhibitors left the synthesis of the other (uninhibited) subunit unaffected for at least four hours. It was, therefore, proposed by these workers that the synthesis of the two subunits is not tightly coupled over short time periods.

Although the experimental data lean somewhat in favour of SSU being necessary for LSU synthesis, the evidence is conflicting.

1.4 *Protein Synthesis during Greening*

The importance of protein synthesis to the development of a mature chloroplast from an etioplast is difficult to ascertain. It is clear that considerable protein synthesis does occur particularly of the membrane proteins (Drumm and Margulies, 1970). However, much of the increase observed in enzymic activity in response to greening is due to enzyme activation rather than *de novo* synthesis. For example, the activity of pyruvate-orthophosphate dikinase increases 15-fold in maize and 8-fold in sorghum when dark grown plants are illuminated for 35 hours. But the same enzyme shows a 20-fold activation when green leaves are moved from dark to light (Graham *et al.*, 1970). The effects of enzyme activation and *de novo* synthesis generally combine to raise the levels of many enzymes.

Adenylate kinase, for instance, will increase three- to four-fold in activity when maize or sorghum leaves are allowed to green. This increase can be inhibited by 60 percent if cycloheximide is used to block protein synthesis, suggesting that both synthesis and activation of adenylate kinase have occurred (Graham *et al.*, 1970).

The increase in enzyme activity during greening is generally two- to three-fold although considerably larger increases have been measured. As examples of the variation that may occur in the extent of activation, triose phosphate isomerase rises by only 40 percent after two days of greening in *Phaseolus vulgaris* (Ireland and Bradbeer, 1971) while in the same plant over the same period ferredoxin-NADP reductase shows a 50-fold increase in activity (Keisler *et al.*, 1962). A comprehensive list of the enzymes that have their activity increased during greening can be found in Kirk and Tilney-Bassett (1978).

There is considerable variation between plant species in the extent of enzyme stimulation by light. In many cases this is due to high pre-existing levels of the enzyme in the etioplast. We find, for example, that ribulose bisphosphate carboxylase will increase 91-fold in activity during greening in peas (Graham *et al.*, 1968) but only three-fold in rye seedlings (Feierabend and Pirson, 1966). In rye etioplasts there are relatively high levels of this enzyme so only a small increase is required to raise levels to those occurring in the mature chloroplast.

In addition to large increases in the activity of soluble enzymes during greening, it was noted at the beginning of this section that considerable synthesis of membrane proteins also occurs. Grebanier *et al.*, (1979) found that during the greening of *Zea mays* seedlings several new membrane proteins that were absent from the

etioplast membranes appeared in the chloroplasts. The most abundant protein of the chloroplast thylakoid, the protein of the light-harvesting pigment-protein complex, showed the greatest increase during chloroplast formation. Some chloroplast-synthesised proteins, such as the two large coupling factor subunits, were present in both etioplasts and chloroplasts in fairly equal amounts. This result agreed with the observation that ATPase activity was largely unaffected by the dark/light transition. Three other proteins were strongly enhanced during greening, 32,000; 16,000; and 13,000 dalton polypeptides, all made by isolated chloroplasts. Grebanier *et al.*, (1979) concluded that most proteins made in the chloroplasts did not have their *in vivo* synthesis stringently regulated by light. A somewhat less comprehensive study by Cobb and Wellburn (1973) gave similar results for greening *Avena* seedlings.

1.5 *RNA Synthesis during Greening*

The change in the pattern of protein synthesis in the leaf during greening is reflected in an alteration in the proportions of the cells' ribosomes associated with messenger RNA molecules to form polysomes. Polysome profiles are frequently taken as a measure of the functional status of the protein synthetic system of the tissue; the higher the proportion of polysomes to free ribosomes the greater the protein synthesis (Davies *et al.*, 1972; Alscher *et al.*, 1978). Therefore, it is no surprise to find that during greening there is a rapid increase in the ratio of polysomes to monosomes (Williams and Novelli, 1968; Malcolm and Russell, 1974; Smith, 1976; Giles *et al.*, 1977; etc). An example of the extent of this change is given by the transfer

of dark grown maize seedlings to the light. In the dark, only 27 percent of the cytoplasmic ribosomes are arranged as polysomes, whereas, after transfer to the light the proportion rises to 84 percent (Travis *et al.*, 1970). It has been difficult to make similar estimates for plastid polysomes due to their apparently, very small size (Chua *et al.*, 1973; Goodenough, 1971; Margulies and Michaels, 1975). We do, however, know that changes occur in the activity of protein synthesis within plastids during their development. This is clear, not only from the data already given on protein accumulation, but also from observations of the RNA content of maturing chloroplasts. The etioplasts contain many ribosomes but in the young radish seedling, for example, there is a doubling of the rate of plastid ribosomal RNA synthesis in response to transfer of dark-grown plants to the light. This increased level of transcription lasts for at least one day (Ingle, 1968). In cotton plants, plastid RNA will rise from 8 to 50 percent of the total leaf RNA during the first five days of greening (Brantner and Dure, 1975). Similar measurements for barley (Smith *et al.*, 1970) and *Vicia faba* (Dyer *et al.*, 1971) showed that plastid RNA changed from 10 to 50 and from 13 to 41 percent of total leaf RNA, respectively. It has also been found that in maize seedlings there is a two-fold increase in the amount of plastid transfer RNA when etiolated plants are given 17 hours of light (Haff and Bogorad, 1976). Even greater increases in plastid tRNA levels have been measured during the greening of cotton seedlings (Merrick and Dure, 1972). It appears, therefore, that the synthesis of both plastid ribosomal and transfer RNAs during greening is a general response to light. In dark grown maize leaves, Harel and Bogorad (1973) found that the first two hours

of light were devoted particularly to the synthesis of plastid RNAs although cytoplasmic RNA also showed a marked increase. Dyer *et al.*, (1971) note that the increased levels of plastid ribosomal and transfer RNAs in *Vicia faba* during greening seem to be essential for full chloroplast differentiation, even though appreciable plastid formation will occur in the absence of this increase.

Unfortunately, there is no clear information available on plastid messenger RNA levels during the development of chloroplasts from etioplasts. In the cytoplasm of maize leaves, RNA is synthesized after about 2½ hours of greening, but it is neither ribosomal nor transfer and is presumed to be mRNA (Harel and Bogorad, 1973). Similarly during the normal development of *Phaseolus aureus* leaves, poly-A-containing RNA is synthesized (Grierson and Covey, 1975). The best evidence for changes in the messenger RNA population of plastids during chloroplast formation has come from measurements of the transcription of *Euglena gracilis* plastid DNA. Rawson and Boerma (1976) found that dark-grown cells initially transcribe about 53 percent of their plastid DNA (7.2×10^4 base pairs), rising to about 57 percent (7.8×10^4 base pairs) after five hours of light and then falling to 47 percent (6.4×10^4 base pairs) at the completion of chloroplast formation. It appeared that in the late stage of chloroplast development the transcribed sequences differed from those transcribed early in development.

Therefore, during the development of chloroplasts from etioplasts there appears to be a change in the regions of the plastid DNA being transcribed. This is probably also true of the nuclear genome since changes also occur in the pattern of nuclear protein synthesis. However,

some messenger RNAs, thought to be nuclear coded, are transcribed in the dark but stored and only activated for translation in response to light (Giles *et al.*, 1977; Smith 1976). There is no evidence to suggest a similar mechanism in plastids. The changes that occur in plastid protein synthesis appear to be accountable for in terms of transcription of messenger and structural RNA (ribosomal and transfer). Plastid gene expression during greening does not seem to be dependent upon the replication of the plastid genome (Bennett and Radcliffe, 1975; Cattolico, 1978), although increases of four-fold have been measured in the amount of plastid DNA during greening (Gibbs *et al.*, 1974). Measurements have not been made of messenger RNA levels during greening to determine whether they may be responsible for the changing rate of protein synthesis in the developing plastids.

1.6 *The Turnover of Proteins during Chloroplast Development*

There are three factors that will affect the increase, or decrease, in the activity of enzymes and other proteins during development. We have already discussed the first two - the involvement of protein synthesis and enzyme activation; the third factor is the rate of protein degradation. Very little is known about the degradation of chloroplast proteins. It is clear that proteolysis plays a very important role in chloroplast development in providing a source of nitrogen and amino acids for new protein synthesis (Harris and Kirk, 1969; Bertini *et al.*, 1965). In *Euglena*, protein breakdown is essential for chloroplast formation since chloroplast development is blocked by protease inhibitors (Zeldin *et al.*, 1973). Transfer of dark-grown plants to the light, therefore, seems to result in proteolysis of proteins not essential for this developmental process.

During chloroplast senescence the reverse situation applies; chloroplast proteins are amongst the first to be degraded. Wittenbach (1978) found that during dark-induced senescence of wheat seedlings the loss of RuBPCase accounted for 80 percent of the total soluble protein lost in the first two days. The proteinases of these seedlings had a particularly high affinity for RuBPCase (Km at 38°C of 1.8×10^{-7} M) as compared with casein (Km 1.1×10^{-6} M). The high affinity of the proteinases for RuBPCase may provide an explanation for the preferential loss of this enzyme during the early (and reversible) stages of senescence in tobacco (Kawashima *et al.*, 1967), *Perilla* (Kannangara and Woolhouse, 1968), barley (Peterson *et al.*, 1973) and wheat (Tung and Brady, 1972).

However, this does not resolve the question of whether the chloroplast proteins, and specifically RuBPCase, are being turned over during chloroplast development or in the mature chloroplast or etioplast. Huffaker and Peterson (1974) discovered that over a 120 hour period there was no turnover of RuBPCase (neither synthesis nor degradation) in barley leaves labelled with $^{14}CO_2$. On the other hand, significant turnover of other soluble proteins could be measured after only 50 hours. In barley and wheat leaves it seems that simultaneous synthesis and degradation (turnover) of RuBPCase does not occur but that it does occur for other soluble leaf proteins (Peterson *et al.*, 1973; Brady and Tung, 1975). Although RuBPCase can be both degraded and synthesised, these processes seem not to occur simultaneously but can be induced independently by changing environmental conditions (Peterson *et al.*, 1973; Zucker, 1971). The stability of RuBPCase is not a property of all chloroplast proteins. Huffaker and Peterson (1974) suggest that phytochrome-controlled enzymes are turned over very rapidly. For example, in barley the chloroplast membrane-bound

enzyme, δ-aminolevulinic acid synthetase (involved in chlorophyll biosynthesis) has a half life of between 10 minutes and 1½ hours (Suzer and Sauer, 1971; Nadler and Granick, 1970). It does not, therefore, appear possible to make a general statement about the stability of chloroplast-localised proteins.

1.7 *Summary*

The formation of mature chloroplasts from etioplasts involves major structural and synthetic changes, mostly to the existing structures and components of the etioplast. Many of the new membrane components of the mature chloroplast appear to be derived almost directly from the prolamellar body of the etioplast. Similarly, most enzymes of the CO_2 fixation cycle are already present and usually increase in activity only two- to three-fold during greening. The rapid conversion of protochlorophyll to chlorophyll allows photosystem I to begin operation within minutes of illumination and although photosystem II responds more slowly, it will have begun operation usually after one hour of light.

Protein synthesis is less rapidly influenced by the onset of light. It usually rises after a lapse of several hours and immediately after ribosomal and transfer RNAs have begun to show a rapid increase in synthetic rate. At the same time there is rapid activation of many photosynthetic and related enzymes. In the chloroplasts new synthesis of structural RNAs is particularly important during greening and appears to be accompanied by the transcription of new sequences of DNA. Although RNA synthesis is important for protein synthesis in the cytoplasm, at least part of the increased synthesis of chloroplast proteins appears to be due to activation of existing messenger RNAs

and the formation of larger, more active polysomes. Despite the very active chloroplast protein synthesis during the greening period, the plastids are still heavily dependent for probably the majority of their proteins on the cytoplasm. However, the chloroplast makes the large subunit of ribulose-bisphosphate carboxylase which can comprise as much as 40-50% of the soluble leaf protein. The chloroplast contribution to leaf protein synthesis is, therefore, quantitatively highly significant.

Most aspects of the regulation of the changeover from etioplast to chloroplast, and also from proplastid to etioplast or to chloroplast, remain unclear. The matter is complicated by the participation of two genomes, nuclear and organellar, and their respective protein synthetic systems. Attempts to identify the coding functions of the two genomes are at a very early stage. In the chloroplast only three or four proteins have been clearly identified with the chloroplast genome. Knowledge of the sites for the synthesis, transport and processing of cytoplasmically synthesised proteins has only recently advanced with the identification of a precursor for the small subunit of RuBPCase. Processing of chloroplast-synthesised membrane proteins also seems probable and in at least one case the processing is dependent upon a cytoplasmic factor. Therefore, it seems that interactions between the nuclear and chloroplast genomes occur at all levels of gene expression, from transcription of the DNA through to processing of the translation products and activation of enzymes.

1.8 *Aims and Scope of the Thesis*

Despite the large amount of data available on the changes that occur in plastid structure and function during development, very little is known about the mechanism that may be responsible for this change. It is clear that many of the changes observed in the plastids are due to protein synthesis. However, the involvement of the plastid genome and plastid protein synthetic machinery is still only poorly understood, particularly when this study was commenced. In this project some of the properties and parameters of plastid gene expression were examined during chloroplast formation and in the mature chloroplast. It was considered that the determination of two parameters of protein synthesis (messenger RNA levels and messenger RNA efficiency) would provide information as to the site and mechanism of regulation of plastid protein synthesis. The role of transcriptional and translational control in regulating gene expression should be revealed by these parameters in conjunction with a knowledge about the rates and types of protein synthesis that are occurring.

In an effort to gain information about the function and influence of the plastid genome on protein synthesis, a study of chloroplast messenger RNAs was also undertaken.

CHAPTER 2

MATERIALS AND METHODS

2.1.1. *Plants*

Pea seeds (*Pisum sativum* L. var. Greenfeast) were surface sterilised in a 7% chlorine solution, imbibed overnight in aerated water and sown in a perlite-vermiculite mixture. The seedlings were kept for 6.5 days in total darkness at 25°C. Etiolated apices were obtained from the dark grown material. For greening experiments the seedlings were transferred, after 6.5 days growth (180 hours after start of imbibition) to light cabinets at 25°C with continuous light at about one third full sunlight (600-700 μ Einsteins/m^2/sec). In all experiments the pea apex was used (the pigmented tip of the seedlings), that is the yellow apical hook of the etiolated plants and the leaves and young growing tip of the green plants.

Wheat seedlings (*Triticum aestivum* L. var. Falcon) were grown in perlite-vermiculite in growth cabinets under a 14 hour day (25°C) and 10 hour night (20°C). The second leaf only was used and sampling was done by cutting the leaf at the ligule various times after the sowing of the dry seeds. Pea and wheat seedlings were watered with nutrient solution every morning and demineralised water every evening.

Spinach plants were grown in liquid culture as described by Spencer and Whitfeld (1967). Young leaves, less than 3 cm long, were used for chloroplast RNA preparations unless otherwise stated. Older leaves were used to extract chloroplast DNA.

2.1.2. *Antibodies, Enzymes and Chloroplast DNA*

Many of the antibody preparations were kindly provided by C.J. Brady (rabbit anti-bean and anti-wheat LSU) and T.J. Higgins (sheep anti-spinach LSU and anti-rabbit immunoglobulin (IgG).

Goat anti-rabbit IgG was obtained from the Commonwealth Serum Laboratories as were Freund's complete and incomplete adjuvants.

The restriction endonucleases used were obtained from Biolabs (Sma I and Hind III) or were kindly provided by I. Oliver (Pst I), J. Langridge (EcoRI) and E. Dennis (Bam I).

I am also particularly grateful to W. Bottomley for the spinach large subunit and total RuBPCase protein and to P.R. Whitfeld for spinach chloroplast DNA.

2.2. *The Isolation of Plastids*

The method used for plastid isolation depended upon the nature of the subsequent experiments. For DNA, RNA and polysome isolation the procedure described by Whitfeld *et al.* (1978) was followed. Intact pea plastids were most efficiently prepared if the tissue was chopped (mechanically or by hand) with a razor blade rather than blended in a Waring blendor (the technique used for spinach leaves).

The plastids used in experiments for radioactive amino-acid incorporation into proteins by isolated plastids (*in organelle*) were isolated in the "Sorbitol" buffer system of Bottomley *et al.*, (1974). Again pea plastids were prepared by chopping and spinach chloroplasts from blended leaves. For light driven incorporation of labelled amino-acids, the plastids were washed and resuspended in the "Sorbitol" buffer containing no $MgCl_2$. The plastids used for ATP driven incorporation were lysed in buffer containing no sorbitol but 10 mM $MgCl_2$, 50 mM Tricine, pH 8.4 (adjusted with KOH), 2 mM EDTA and 4 mM β-mercaptoethanol.

2.3. *The Preparation of Polysomes*

Polysomes were extracted from total leaf and apical tissue according to the method of Davies *et al.*, (1972) and dissolved in 20 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, and 20 mM KCl. The polysomes were stored under liquid nitrogen.

Plastid polysomes were obtained from plastids isolated by the methods just described (section 2.2). Membrane-bound polysomes were obtained from chloroplast membranes prepared by lysing chloroplasts in a hypotonic buffer, collecting the membranes by centrifugation at 5,000 rpm for 5 minutes and washing four times in the buffer given above. The membranes were solubilised in 5 percent Triton X-100 and the polysomes collected by centrifugation at 65,000 rpm for 1.5 hours in the Ti75 rotor (Beckman L5-65 centrifuge) through a 50 percent sucrose cushion. Free polysomes were obtained from the supernatant fractions of the membrane washings. Lysis of chloroplasts directly, with 5 percent Triton X-100, provided the total plastid polysome fraction.

Where chloramphenicol was added to the buffers during chloroplast polysome isolation, a concentration of 50 µg per ml was used.

"The hybrid polysomes", described in Chapter 4, of *E. coli* ribosomes on spinach chloroplast mRNA were prepared from an *E. coli* cell-free S-30 assay mix (section 2.9.2). Chloroplast RNA was added to the *E. coli* S-30 to give 1 mg RNA per ml. After 10 minutes incubation at 37°C, chloramphenicol was added (final concentration 50 µg/ml)) and the assay mix cooled on ice. Polysomes were collected by centrifugation for 1 hour at 50,000 rpm at 4°C. The ribosome pellet was dissolved in 10 mM Tris-HCl, pH 8.5, 140 mM

NaCl and 5 mM EDTA. This dissociated the ribosomes and the preparation was ready for sucrose gradient fractionation (section 2.10).

2.4. *RNA Isolation*

Plastids were prepared from peas and spinach as described earlier (section 2.2) and lysed with 5 percent Triton X-100 in 10 mM Tris-HCl, pH 8.5, 50 mM KCl and 10 mM $MgCl_2$. Contaminating nuclei were pelleted by centrifugation at 3,000 rpm for 5 minutes and the supernatant was digested for 20 minutes on ice with deoxyribonuclease (Boehringer, bovine pancreas, grade II) at 20 µg per ml. At the end of digestion sodium-dodecyl-sulphate (SDS) was added to 2 percent as well as an equal volume of phenol (10 mM Tris-HCl, pH 7.5, buffer saturated). Following two extractions with phenol the RNA was precipitated from the aqueous phase with two volumes of ethanol and 0.1 volume of 20 percent sodium acetate pH 5.5. After at least two hours at -20°C the RNA was recovered by centrifugation, washed with 70 percent ethanol, dried, redissolved in distilled water and stored at -20°C.

The preparation of RNA from spinach chloroplasts for subsequent messenger fractionation required that the RNA solutions be kept cold. If allowed to warm, even slightly, the 23S chloroplast ribosomal RNA would break down. In order to minimise problems due to ribonuclease all solutions were treated with diethyl-pyrocarbonate (where possible), plastic ware was soaked in 0.5 N potassium hydroxide and glassware was autoclaved for 1 to 1.5 hours.

Total pea apex RNA was prepared from apices powdered in liquid nitrogen. The fine powder was allowed to thaw in 50 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$, 60 mM NaCl, 5 mM EDTA and 5 percent Triton X-100. After grinding with a mortar and pestle, the cell debris was centrifuged out and the supernatant digested with deoxyribonuclease as above.

The solution was deproteinised by two extractions with SDS-phenol and the RNA recovered by ethanol precipitation. These RNA preparations were used to determine the proportion of plastid to cytoplasmic RNA during greening (see next section, 2.5).

Estimation of the total RNA and DNA content was carried out on nucleic acid extracts of the pea apices prepared, essentially, as described by Rijven and Williams (1965). The apices were held intact through a series of extractions with ethanol, perchloric acid, n-butanol and diethyl ether, to remove most soluble substances except nucleic acids. The nucleic acids were then hydrolysed out of the tissue with hot perchloric acid. The technique of Burton (1956) was used to estimate the DNA content of the extracts and by subtraction from the total nucleic acid content (estimated from the 260 nm absorbances), the amount of RNA per apex was estimated. Techniques for the direct assay of RNA in the extracts by the modified orcinol procedure (Almog and Shirey, 1978) were not successful due to orcinol-reactive contaminants in the extracts.

2.5. *Analytical Gel Fractionation of RNA.*

The estimation of the proportion of plastid to total RNA was achieved by the analysis of total RNA tissue extracts (prepared as above) on 2.2 percent (w/v) polyacrylamide gels. Gels were made in perspex tubes (9 x 0.6 cm) and contained 36 mM Tris-HCl, 30 mM sodium phosphate, 1 mM EDTA, pH 7.0 (Leaver, 1973) plus 0.1 percent SDS. After electrophoresis at room temperature for 2½ hours (5 mA per gel), the gels were washed for at least 30 minutes in 20 percent ethanol and then scanned at 260 nm. The size of the absorption peaks was used to quantitate specific RNA size classes. The ratio of 16S to 18S RNA gave an estimate of the proportion of plastid to cytoplasmic RNA.

Band locations were verified by staining the gels with 0.05 percent Toluidine Blue in 20 percent ethanol.

RNA preparations were also analysed under denaturing conditions on agarose gels in the presence of urea. The gels (15 cm x 20 cm x 0.33 cm containing sample wells 1 x 0.3 x 0.2 cm) were composed of 1.7 percent (w/v) agarose in 6 M urea and 25 mM sodium acetate, pH 5.0 and required 2 hours at 4°C to set. Sponge plastic (Wettex) wicks were used to provide electrical contact between the gel and two 1 litre buffer tanks (containing 25 mM sodium acetate, pH 5.0).

RNA samples were prepared in 6 M urea plus 5 percent sucrose (ribonuclease free) to give a volume of about 40 µl. After heating at 70°C for one minute, the samples were cooled in iced water and loaded into the gel. Acid fuchsin (1 µg per 40 µl sample volume) provided a useful marker dye since it migrated just ahead of the 4S RNA. The gels were run at about 200 volts for 4 to 5 hours at 4°C. RNA bands on the gels were revealed under ultra-violet light after staining with ethidium bromide (1 ml of a 0.05 percent stock solution in 100 ml H_2O) for 15 minutes.

2.6.1. *Preparative Urea-Agarose Gel Electrophoresis of Spinach Chloroplast RNA*

Preparative urea-agarose gels were made in much the same way as the analytical gels described earlier (Section 2.5). In a sterile glass 250 ml flask 54 g Urea (Schwarz-Mann, ultra-pure), 0.23 ml acetic acid and about 120 ml water (sterile) were mixed. The pH of the solution was adjusted to 5.0 with NaOH. To this was added 2.5 g agarose (Sigma) and 0.20 ml diethylpyrocarbonate. The solution was

heated until it just began to boil; it was then cooled at room temperature for about 30 minutes with constant stirring. The solution was then made up to 150 ml.

The gel (15 x 20 cm x 0.5 cm) was poured in the cold room and allowed 2 hours to set. A 3 mm thick strip of perspex was used to form one long well. The gel was usually pre-run at 200 volts for at least 30 minutes. The sample was prepared in 6 M urea and 10 percent sucrose to give a volume of 0.8 ml and after heating to 70°C for 1 minute and cooling on ice, was loaded in the well under running buffer. After running the sample into the gel for about 1 hour at 75 to 100 volts the voltage was increased to 200 to 250 volts and the sample electrophoresed for a further 6 to 7 hours. The running buffer contained 25 mM sodium acetate, pH 5.0 and was connected to the gel via sponge plastic (Wettex) wicks (sterile).

All perspex was treated with 0.5 N KOH and all glassware heat sterilised.

After electrophoresis, 1 cm wide strips were cut off the side of the gel and stained for 15 minutes in ethidium bromide (1 ml of a 0.05 percent solution in 100 ml water). This permitted localization of the major RNA bands approximately. The gels could provide good resolution for up to 1 mg of chloroplast RNA.

2.6.2. *Extraction of RNA from Urea-Agarose Gels*

The recovery of RNA from the gel was achieved by the butanol-quaternary ammonium ion (QN^+) extraction method outlined in Chapter 4. This technique, also suitable for the recovery of DNA from agarose gels with over 90 percent recovery, is described in (Langridge *et al.*, in press).

The gels were cut into slices of equal size and the volume of each slice was estimated. The urea-agarose was melted in glass tubes at 75°C for 2 minutes and allowed to cool to room temperature. The presence of the urea in the gel kept the agarose molten at 25°C for up to 2 hours. To each tube an equal volume of butanol $+QN^+$ and water (saturated with butanol $+QN^+$) was added (the preparation of these solutions is described below). The solutions were mixed by inversion about 100 times. The phases were separated by centrifugation at 3,000 rpm for 3 minutes at room temperature. The upper phase (butanol) was removed. To the lower phase another volume of butanol was added (butanol saturated with water but containing no QN^+). Again the solutions were mixed by inversion and the phases separated by centrifugation. The extraction was repeated with butanol (containing no QN^+). The butanol phases were pooled and extracted twice with 0.2 M NaCl (2 ml per ml original gel solution). The lower phases (NaCl) were collected, cooled on ice and most residual QN^+ was removed by adding dropwise an equal volume of chloroform to the ice cold tubes. The upper phase (aqueous) was retained. RNA was recovered from this phase by ethanol-sodium acetate precipitation overnight at -20°C in polyallomer tubes. The RNA was pelleted by centrifugation for 1 hour at 40,000 rpm in the SW 41 rotor at -5°C. After washing with 70 percent ethanol the RNA was dried, dissolved in water, and stored at -20°C. The time required for extraction, from melting the gel to the start of the ethanol precipitation, was about 1 hour for 12 gel slices.

The two-phase butanol solution was prepared essentially as described by Hurst and Sheng (1976). 1-butanol (150 ml) and glass-distilled water were equilibrated by shaking in a separating funnel.

The phases were separated and 5 g hexadecyl-trimethyl ammonium bromide (QN^+) was dissolved in 150 ml of the butanol fraction which was then shaken with 100 ml of the equilibrated aqueous phase (from above). Antifoam A (Sigma) (50 µl) was added to reduce the formation of emulsions. The solution was left overnight for phase separation, when each was bottled separately and stored at room temperature.

Other techniques used for recovering RNA from the urea-agarose gels were based upon published techniques; electroelution (Wienand *et al.*, 1979), freeze-squeeze (Thurling *et al.*, 1975; Dolja *et al.*, 1977) and extrusion through a syringe (Amalric *et al.*, 1978).

2.7. *SDS-Polyacrylamide Gel Electrophoresis of Proteins*

Polypeptides were separated on slab acrylamide gels (16 x 18 x 0.15 cm) of either a single acrylamide/bis-acrylamide concentration or a gradient of acrylamide. The single percentage gels were used to separate *in vitro* translation products (section 2.9.2) where resolution in the low molecular weight region (less than 30,000 daltons) was not essential. Gradient gels were used for the separation of *in organelle* and *in vivo* products (sections 2.9.1 and 2.9.2) since these gels gave good resolution over a broad molecular weight range. The relationship between polypeptide molecular weight and mobility on the gels was approximately log-linear. Unless stated on the figure, the gels used were of the single percentage type.

Single percentage acrylamide gels (14 percent acrylamide and 0.19 percent bis-acrylamide) were prepared as described by Higgins *et al.* (1976). Gradient gels were prepared by modification of the method of Laemmli and Favre (1973) (Higgins, personal communication). The gradients were produced using a device with two equal-sized

chambers connected via a tube at their base, and a peristaltic pump (flow rate, 10 to 15 ml per minute). The heavy solution (in the mixing chamber) was pumped out first into the gel mould, keeping the tip of the out-flow 1 to 2 cm above the top of the developing gradient. The heavy solution consisted of 16.7 ml stock acrylamide (30 percent acrylamide and 0.4 percent bis-acrylamide, w/v), 6.25 ml of 1.5 M Tris-HCl, pH 8.4, 2.5 g glycerol, 0.25 ml 10 percent SDS (w/v), 36 µl ammonium persulphate (10 percent w/v, freshly prepared) and 7 µl N,N,N',N'-tetramethylethylene diamine (TEMED). The light solution contained 8.33 ml stock acrylamide, 6.25 ml 1.5 M Tris-HCl, pH 8.4, 10.1 ml water, 0.25 ml 10 percent SDS, 85 µl 10 percent ammonium persulphate and 18 µl TEMED. The resulting gradient was linear from 10 to 20 percent acrylamide.

Both single percentage and gradient gels were overlaid with a stacking gel. The stacking gel consisted of 3.2 ml stock acrylamide, 2.0 ml 1 M Tris-HCl, pH 6.8, 10.5 ml water, 0.16 ml 10 percent SDS, 0.1 ml 10 percent ammonium persulphate, and 15 µl TEMED.

The upper and lower reservoir running buffers contained 1 percent SDS, Tris, 3 g/l, and glycine, 14 g/l. Electrophoresis was overnight at room temperature; 200 mA-hours per single percentage gel and 250 mA-hours per gradient slab gel.

The gels were fixed in 7 percent acetic acid plus 25 percent ethanol and most SDS was washed out of the gel before staining with 0.5 percent Coomassie Brilliant Blue R (Sigma) in 10 percent acetic acid plus 50 percent ethanol (stained for 15 to 30 minutes). The gels were destained by diffusion in the fixing solution and prepared for fluorography as described by Bonner and Laskey (1974) and Laskey and Mills (1975). Partial dehydration of the gels in

saturated polyethylene glycol 6000, reduced the size of the gels and enhanced handling, drying and subsequent fluorography. The gels were dried under vacuum and placed in contact with pre-fogged Kodak X-Ray film RP54 for varying periods (2 to 21 days) at -80°C. In some cases, before the technique of fluorography was developed, gels were simply autoradiographed. Photographs of autoradiographs are indicated.

Samples for electrophoresis on acrylamide gels were prepared by precipitation with 1.5 volumes of methanol plus 6 µl/ml acetic acid at -20°C overnight (for dilute samples) or with 9 volumes of cold acetone at -20°C for 1 to 4 hours. Samples were dissolved in 50 µl of 0.125 M Tris-HCl, pH 6.7, 2 percent SDS, 1 percent β-mercaptoethanol, 5 percent glycerol, and 0.1 percent Bromophenol Blue. Before loading, the samples were heated to 80°C for 2 minutes. Marker proteins used for the estimation of molecular weights were, bovine serum albumin (68,000 daltons), LSU or RuBPCase (55,000 daltons), ovalbumin (45,000 daltons), chymotrypsinogen (25,000 daltons) and the SSU of RuBPCase (14,000 daltons).

2.8. *Estimation of Radioactivity Incorporated into Specific SDS-Polyacrylamide Gel Separated Polypeptides*

For a number of experiments the radioactivity of ^{35}S-methionine incorporated into LSU and other polypeptides was estimated. For this purpose gels were fluorographed as described (section 2.6) and theregions of the dried gels corresponding to the bands of interest were cut out and placed in 5 ml glass vials. Each gel piece, measuring up to 1.5 x 0.5 cm, was allowed to swell in 0.5 ml water for about 2 hours. When all the water had been absorbed by the gel piece, 1 ml of Soluene-350 (Packard, 0.5 N quaternary ammonium

hydroxide in toluene) was added to each vial. The vials were capped and incubated at 50°C until the PPO in the gels (from the fluorography preparation) was completely dissolved, the gel pieces becoming transparent. Scintillation fluid was then added to fill each vial (6 g 2,4-diphenyl oxazole, 0.6 g 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene and 85 ml Triton X-100 per litre of toluene).

The samples were allowed to swell overnight before counting. The counting efficiency was found to be consistently between 60 and 65 percent for ^{35}sulphur.

2.9. *Protein Synthesis Assays*

2.9.1. In vivo *Labelling of Pea Apices*

Only two developmental times were used for *in vivo* labelling; etiolated peas and peas greened for 20 hours. The apices, usually four for each time point, were immersed in 20 mM potassium acetate, 0.1 percent β-mercaptoethanol containing L-^{35}S-methionine (1 m Ci/ml) final concentration. About 200 µl radioactive buffer solution was used for ten to twenty apices. The apices were incubated in the label for various times under lights in the growth cabinet at 25°C (for the 20 hour peas) and in the dark at 25°C (for the etiolated apices). At each time point one apex was powdered under liquid N_2 followed by extraction of the proteins in 800 µl of buffer containing 20 mM TES [N-*tris*(hydroxymethyl)methyl-2-aminoethanesulfonic acid], pH 7.8, and 500 mM NaCl. The supernatant obtained after centrifugation at 8,000 rpm for 5 minutes was acetone precipitated. This fraction provided the total protein extract from the apices.

Plastid proteins were prepared from pea plastids isolated essentially as described earlier for the preparation of plastids for RNA extraction (section 2.2). The apices were chopped by hand with a razor blade in one volume of buffer, filtered through two layers of miracloth

and the plastids collected by centrifugation for 40 seconds in a Beckman Microfuge B. The plastids were washed twice prior to lysis with Triton X-100 (5 percent w/v). Insoluble matter was centrifuged down and the supernatant acetone precipitated to provide the plastid protein extract.

Aliquots of both the total and plastid extracts were taken prior to precipitation for estimating radioactivity taken up by the apices and plastids, and the radioactivity incorporated into TCA precipitable protein.

2.9.2. *Protein Synthesis by Isolated Plastids* (In organelle)

The plastids were isolated according to the procedures given earlier (section 2.2). The incorporation of ^{35}S-methionine into protein by isolated plastids of pea and spinach plants was essentially as described by Bottomley *et al.*, (1974) with only two modifications; firstly, ^{35}S-methionine (Radiochemical Centre, Amersham, 10 µCi per 100 µl assay) was used in place of a ^{14}C-amino acid mixture and secondly, at the end of the incubation 5 or 10 µl of reaction mixture was assessed for TCA precipitable counts by the method of Bollum (1968). Generally, incubation of the plastids for *in organelle* labelling was at 25°C for 30 minutes. However, for transit time measurements, samples were taken at 30-second or 1-minute intervals. These short time samples were frozen immediately in liquid N_2 for subsequent processing (see Section 2.13). The longer 30-minute incubations were terminated by cooling the tubes in ice if the plastids were to be further processed (see below), or by the addition of 9 volumes of cold acetone if total plastid protein was required.

Assay mixes that were to be used to yield membrane and soluble protein fractions were diluted with buffer containing no osmoticum (50 mM Tricine, pH 8.4, 10 mM β-mercaptoethanol, 10 mM $MgCl_2$). For the light driven assays, using intact plastids, this buffer lysed the plastids and released soluble protein. The plastid membranes were centrifuged down and washed 4 times with the above buffer. The membranes were then dissolved in 5 percent Triton X-100 (buffered as above) and after centrifugation to remove insoluble particles, the supernatant was acetone precipitated (membrane proteins). The supernatant fraction from the washing of the membranes was acetone precipitated to yield the soluble plastid protein fraction.

2.9.3. In vitro *Translation of Plastid Messenger RNAs and Polysomes*

The cell-free, *E. coli*, 30,000 x g supernatant, translation system (*E. coli* S-30) was prepared as described by Bottomley and Whitfeld (1979). It was, however, found that rapid dialysis for only 3 to 4 hours, against 6 one litre changes of buffer, gave a more active *E. coli* S-30 preparation.

The translation assays (50 µl assay volume) contained 2 µl salts (1.2 M Tris-acetate, pH 8.15, 1.5 M NH_4Cl), between 0.8 and 1.5 µl 0.5 M magnesium acetate, 2.5 µl energy (3 mM ATP, 0.5 mM GTP and 5 mM phospho*enol*pyruvate), 1 µl of a mixture of 2.5 mM 19 essential amino acids (no methionine), 1 µl 1 M dithiothreitol, 5 µl ^{35}S-methionine (1 m Ci per ml) and between 2 and 7 µl *E. coli* S-30 (depending upon the activity of the preparation). The optimal concentration of magnesium acetate was determined for each new *E. coli* S-30 preparation. It was found to be critical to have the magnesium concentration optimum determined to within 1 or 2 mM for assays to be reliable.

Depending upon the activity of the mRNA preparation between 0.1 and 200 µg of RNA were added to a 50 µl translation assay. Incubation was allowed to proceed for 20 minutes at 37°C unless stated otherwise. At the end of the incubation 5 µl of the reaction mix were spotted onto Whatman 3 mm filter paper and washed by a method modified from Bollum (1968). The papers were washed in 10 percent trichloroacetic acid (TCA) followed by 15 minutes washing in 5 percent TCA at 90°C and 2 washes at room temperature. Finally the papers were washed with a 50 percent ethanol/ether mix and then with ether before drying and counting. The protein in the remainder of the reaction mixture was precipitated by the addition of 2 ml acetone and prepared for SDS-polyacrylamide gel electrophoresis.

When plastid polysomes were used to provide the template for "run-off" translation the assays were exactly as described above for plastid RNA except that aurin tricarboxylic acid (ATA) (20 to 30 µm) was generally added to prevent the ribosomes from re-initiating protein synthesis. Polysomes tended to be more active than comparable amounts of purified RNA in incorporating ^{35}S-methionine into protein *in vitro*. Usually 2 to 5 µg of polysomes gave levels of ^{35}S-methionine incorporation equivalent to 20 to 40 µg of plastid RNA.

2.10 *Sucrose Gradient Centrifugation*

Isokinetic sucrose density gradients were used almost exclusively for the fractionation of polysomes, ribosomes and ribosomal subunits, and messenger RNAs. The calculation and construction of the gradients are described in Appendix I. The nature of the gradients used for the various fractionation purposes can be described by a number of parameters given in Table 2.1. The gradient parameters set out in Table 2.1 relate to the variables given in Appendix I.

Particle	Particle Density (g/cc)	Centrifugation rotor speed (rpm x 10^3)		Gradient v (m)	C_i (%)	C_r (%)	Time (hours)
Polysomes	1.41	SW 41	41	8.12	10	29	1.5
		SW 50.1	50	3.68	15	27	0.75
Ribosomal subunits	1.41	SW 41	41	8.12	10	29	6.75
RNA	1.9	SW 41	41	5.89	10	30.5	18.5

All gradients were run at 5°C

Table 2.1 Sucrose gradient parameters for various fractionation functions. (see Appendix I for explanations of "v", "C_i", "C_r" and "time").

After centrifugation the gradients were pumped from the top of the tubes through a 0.5 cm light path flow cell of an Isco Model UA-5 Absorbance monitor set at 260 nm and the absorbance profile recorded. Where fractions were taken from a gradient, the location of each fraction on the absorbance profile was recorded.

The gradient buffers for polysome fractionation contained 20 mM Tris-HCl, pH 8.5, 10 mM $MgCl_2$ and 20 mM KCl. The gradients used for fractionating ribosomal subunits contained no $MgCl_2$ but 5 mM EDTA. RNA fractionation was carried out in several different buffers;

(a) Mg^{2+} buffer - 50 mM Tris-HCl, pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$.

(b) No Mg^{2+} buffer - 50 mM Tris-HCl, pH 8.5, 100 mM NaCl, 20 mM KCl.

(c) LiOAc buffer - 0.15 M lithium acetate, pH 6.0.

The LiOAc buffer was used most extensively (see Chapter 4). The type of gradient buffer used for fractionation is indicated in the text or figures.

2.11. *Buoyant Density Gradient Fractionation of Spinach Chloroplast Ribonucleoprotein*

Spinach chloroplast ribonucleoprotein was prepared by dissociating chloroplast polysome preparations in buffer containing 5 mM EDTA. Ribonucleoprotein particles were fractionated on caesium sulphate gradients as described by Greenberg (1977) and Liautard and Liautard (1977) or on metrizamide gradients (Buckingham and Gros, 1975). Fractions were collected and RNA prepared by phenol extraction and precipitation for *in vitro* translation assays.

2.12 *Estimation of the Mean Size of Polysomes Synthesising Specific Polypeptides*

2.12.1 *Polysome Size Estimation by Sucrose Gradient Fractionation and "Run-off" Assays.*

Free and membrane-bound pea plastid polysomes were prepared from each developmental time as described earlier (section 2.3). After separation on isokinetic sucrose gradients (section 2.10) six fractions were collected from each gradient and the polysomes recovered from the fractions by centrifugation at 65,000 rpm for 1.5 hours in the Ti 75 rotor at 2°C. The *E. coli* S-30 assay mix was added directly to each centrifuge tube, mixed vigorously, and incubated at 37°C for 20 minutes with constant gentle agitation. The products of *in vitro* runoff were assessed by gradient SDS-poly-acrylamide gel electrophoresis as described earlier (section 2.7).

The radioactivity incorporated into specific proteins by the polysome gradient fractions was measured (section 2.8) and the distribution of polysomes synthesising the polypeptide of interest was estimated.

The mean polysome size for any polypeptide can be calculated using the following equations:

$$\frac{\Sigma(x \cdot \bar{S}_x)}{\Sigma x}$$ = mean sedimentation velocity based upon activity alone (PA).

$$\frac{\Sigma \frac{(x \cdot \bar{S}_x)}{\bar{P}x}}{\Sigma x}$$ = corrected mean sedimentation velocity (Pc).

where x = radioactivity incorporated into polypeptides by the runoff assay of one fraction from the sucrose gradient.

$\bar{S}_x$ = mean sedimentation velocity of the fraction.

$\bar{P}_x$ = mean polysome size of the fraction (number of plastid ribosomes).

The values given in Chapter 3 have been corrected for the effect of polysome size on activity (P_c). In order to express the results in terms of the mean number of ribosomes bound to the messenger RNA of interest, the sedimentation velocity can be correlated to standard plots of sedimentation velocity versus polysome size (from the sucrose gradient profile). Figure 2.1 shows this relationship for plastid and cytoplasmic polysomes.

2.12.2. *Immunological Identification of LSU Synthesising Polysomes.*

The preparation of antibodies against LSU is described in section 2.14. Three types of conjugates were used to label the antibodies; ferritin, ^{125}-iodine and fluorescamine.

2.12.2 (a) Preparation of ferritin-antibody and labelling of polysomes.

All solutions and glassware were treated to prevent ribonuclease contamination of the final ferritin-antibody preparation.

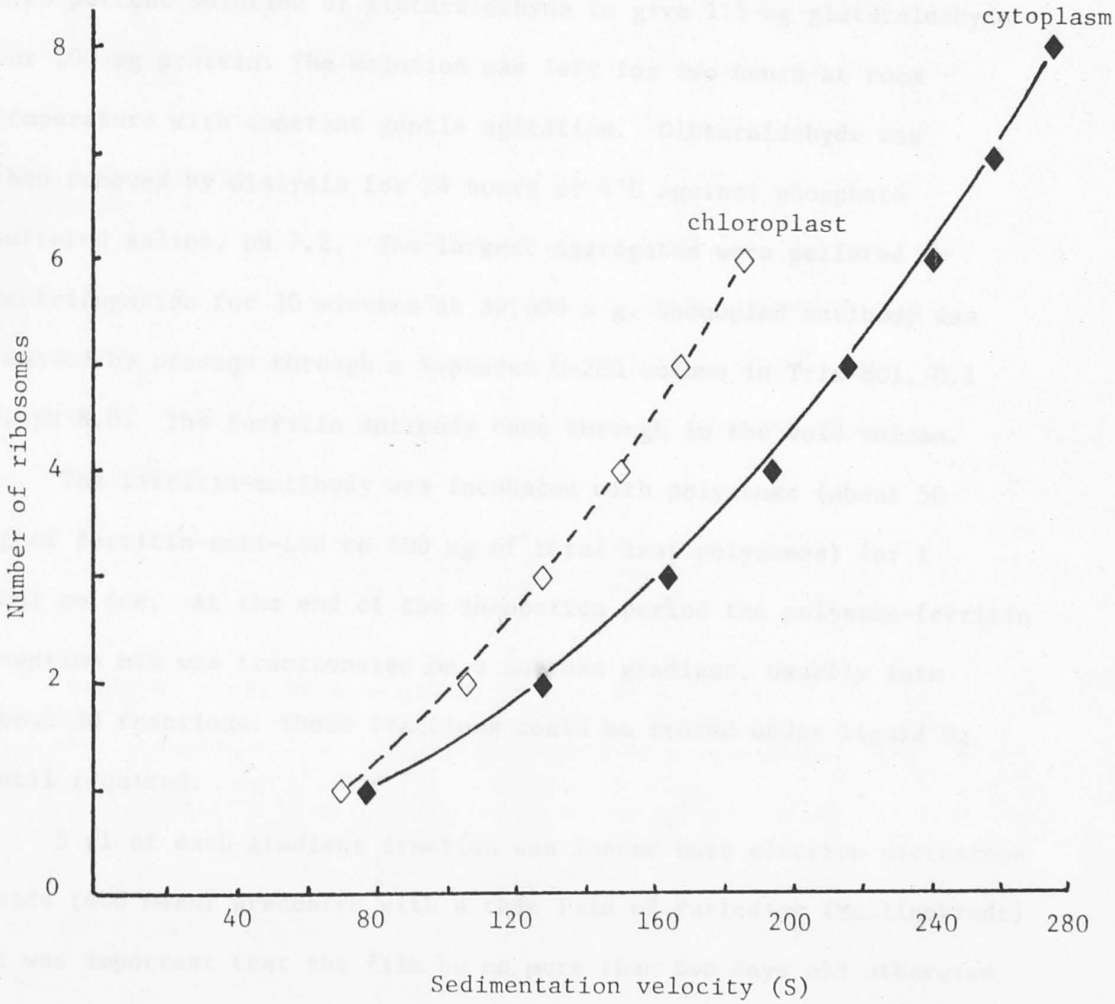


Figure 2.1. Sedimentation velocity of chloroplast and cytoplasmic polysomes.

Antibodies were coupled to ferritin using the glutaraldehyde technique of Neauport-Sautes and Silvestre (1972). 40 mg crystallised ferritin (equine, Calbiochem grade A) was mixed with 10 mg of antibody protein in 0.1 M sodium phosphate buffer at pH 6.8 to give a concentration of protein of 25 mg per ml. To this was added a 0.25 percent solution of glutaraldehyde to give 1.5 mg glutaraldehyde per 100 mg protein. The solution was left for two hours at room temperature with constant gentle agitation. Glutaraldehyde was then removed by dialysis for 24 hours at 4°C against phosphate buffered saline, pH 7.2. The largest aggregates were pelleted by centrifugation for 30 minutes at 30,000 x g. Uncoupled antibody was removed by passage through a Sephadex G-200 column in Tris-HCl, 0.1 M, pH 8.0. The ferritin antibody came through in the void volume.

The ferritin-antibody was incubated with polysomes (about 50 µg of ferritin-anti-LSU to 400 µg of total leaf polysomes) for 1 hour on ice. At the end of the incubation period the polysome-ferritin reaction mix was fractionated on a sucrose gradient, usually into about 30 fractions. These fractions could be stored under liquid N_2 until required.

5 µl of each gradient fraction was loaded onto electron microscope grids (400 mesh) precoated with a thin film of Parlodion (Mallinckrodt). It was important that the film be no more than two days old otherwise the ribosomes did not spread well on the grid. After one or two minutes on the grid the surplus solution was removed. The grids were then washed through an ethanol series of 50 to 95 percent (v/v) ethanol. The surface film of the grid was strengthened, after washing, with a layer of carbon.

The proportion of ribosomes labelled with the ferritin antibody was scored using an electron microscope at a magnification of 40,000 to 45,000 times.

For many of the fractions, particularly those from the top of the gradient, it was necessary to dilute the ferritin-labelled polysome solutions 10 or even 100 fold to obtain usable densities of ribosomes on the microscope grids. The technique has been used to assess polysome sizes with as little as 10 µg of a total wheat leaf polysome preparation (containing only 2 to 3 µg of chloroplast polysomes). It is, however, important to correct for non-specific binding and random coincidence of ferritin and polysomes on the microscope grid since these factors can be highly significant (see Chapter 3). Wheat root polysomes were used to provide an estimate of this background.

2.12.2 (b) ^{125}Iodine-anti-LSU labelling of total wheat leaf polysomes.

Antibody preparations were iodinated by a method of Higgins (personal communication) using the chloramine-T procedure. The antibody solution (30 µg protein in 15 µl phosphate buffer, pH 7.0) was added to 10 µl ^{125}Iodine (10 µCi/µl) and the reaction started with 3 µl Chloramine-T (5 mg/ml in phosphate buffer). The reaction was allowed to proceed for 15 minutes at room temperature. The addition of 4 µl metabisulphite (5 mg/ml), 2 µl 1M potassium iodide and 20 µl BSA (5 mg/ml) plus 1 ml 10 mM sodium phosphate buffer pH 7.0, and 10 mM NaCl, stopped the reaction. The preparation was then dialysed for 3 days against 10 mM sodium phosphate pH 7.0, 10 mM NaCl and 0.1 percent (w/v) sodium azide.

The ^{125}I-anti-LSU was incubated with polysomes as outlined above for the ferritin-anti-LSU. After sucrose gradient fractionation the distribution of radioactivity down the gradient was measured by scintillation counting.

2.12.2 (c). Fluorescent-anti-LSU labelling of total wheat leaf polysomes.

Fluorescent labelled rabbit anti-bean LSU was kindly provided by Colin Brady (CSIRO, Plant Physiology Unit). The labelling of wheat leaf polysomes was as described above. This technique did not give sufficiently strong labelling to allow detection of the antibody down a sucrose gradient.

2.13. *Estimation of Ribosome Transit Times*

The method used to calculate transit times for plastid ribosomes is based upon the technique of Fan and Penman (1970). The principle behind this technique is described in Chapter 3 (Introduction). The incorporation of radioactive ^{35}S-methionine by isolated pea plastids into polypeptides released from ribosomes and into total plastid protein (ribosome-bound plus released protein) was measured over a five to six minute time course.

Broken pea plastids were allowed to incorporate ^{35}S-methionine into protein in an ATP driven *in organelle* translation system (section 2.9.2). Samples of 50 µl were taken from the assay every 30 or 60 seconds and frozen in liquid N_2. When convenient the frozen samples were thawed in 0.5 ml Tris-HCl, pH 8.5 (20 mM), KCl (50 mM), $MgCl_2$ (10 mM) and Triton X-100 (5 percent, w/v). The samples were centrifuged at 5,000 rpm for 5 minutes to pellet insoluble material, and 200 µl was spotted onto filter discs (Whatman 3 MM) (total incorporation) and the remainder centrifuged

at 35,000 rpm for 3 hours at 2°C to pellet ribosomes. The supernatant of this centrifugation step was also dried onto filter discs (incorporation into released protein). The filter discs were washed in TCA as described earlier (section 2.9.3). As explained in Chapter 3, the time difference between the graphs of total and released incorporation is equal to one-half the mean plastid ribosome transit time.

The transit time for ribosomes synthesising the LSU protein was estimated by separating released and total plastid proteins on SDS-polyacrylamide gels and counting the radioactivity incorporated in LSU alone (see Section 2.8).

2.14 *Preparation of Antibodies*

Antibodies were prepared in rabbits against spinach-LSU and against sheep IgG. Spinach LSU was purified by W. Bottomley. The rabbit antibodies were prepared by sonicating 2 mg protein in 50 mM Tris-HCl, pH 9.0, 200 mM NaCl and an equal volume of Freund's complete adjuvant The emulsion was injected subcutaneously at multiple sites in the hind leg muscles. This was repeated twice at 14 day intervals and at the end of this period blood samples were taken every week for testing. When suitable antibody levels appeared in the serum, 30 ml of blood were taken at weekly intervals. During the bleeding period the rabbits were given booster injections of 0.5 mg protein in Freund's incomplete adjuvant.

IgG was purified from the serum by triple ammonium sulphate precipitation according to the method of Hebert *et al.*, (1973). Ribonuclease was removed from antibody preparations destined for use with polysomes (for immunoprecipitation or labelling) by passage through carboxymethyl-cellulose and diethyl-aminoethyl-

cellulose as described by Palacios *et al.*, (1972). The presence of ribonuclease in the antibody preparations was assayed by incubating the preparations with polysomes and determining the effects on sucrose gradient profiles. Concentrations of pancreatic ribonuclease as low as 10^{-17} gm per ml could be detected by this method.

2.15. *Immuno-Fractionation Techniques*

2.15.1. *Immunochromatography of* in vitro *Translation Products of Spinach Chloroplast RNA.*

Antibodies made against LSU were coupled onto cyanogen bromide activated Sepharose 4B (Pharmacia) according to the procedure recommended by the manufacturer. The total *E. coli* S-30 reaction mixture, containing ^{35}S-methionine labelled translation products, was loaded onto the column in phosphate buffered saline (pH 7.2). The column was washed thoroughly with buffer and non-specific binding was reduced by washing with 3 M NaCl. The bound protein was released with 8 M Urea. Other chaotropic eluants such as ammonium thiocyanate and acetic acid were also tested, but gave similar results.

The eluted protein was freed of urea by dialysis and then precipitated with acetic-methanol as described earlier (section 2.7). Dialysis and precipitation were done in the presence of purified LSU to act as carrier.

2.15.2. *Immunoprecipitation of LSU Synthesising Polysomes.*

A range of techniques for indirect immunoprecipitation was used. These techniques are indicated in Table 2.2.

First Antibody	Secondary Antibody or precipitating factor	Reference to technique followed.
Rabbit anti-bean LSU	Goat anti-Rabbit IgG	Shapiro *et al.* (1974)
" " -wheat LSU	Sheep " " "	" " " "
" " -spinach LSU	*Staphayloccus aureus* protein A	Kessler (1975) and N. Gough (personal communication)
Sheep " " LSU	Rabbit anti-Sheep IgG	Shapiro *et al.* (1974)

Table 2.2. Indirect immunoprecipitation techniques tested for LSU polysome precipitation.

Antibody preparations, purified by affinity chromatography against LSU linked via cyanogen bromide to Sepharose 4B, were also tested as the primary antibody.

2.15.3. *Immunochromatography of LSU Synthesising Polysomes.*

The techniques used for immunochromatography of wheat and spinach polysomes were based upon the coupling of anti-rabbit IgG to Sepharose 4B or to cellulose. The Sepharose 4B coupling procedure used cyanogen bromide activated Sepharose 4B (Pharmacia). Coupling of antibodies to cellulose followed the method described by Schutz *et al.*, (1977) with linkage via diazotization. Both p-aminobenzyl cellulose (Servacel, pab 23) and m-aminobenzyloxymethyl cellulose (Miles-Yeda Ltd) were tested. The preparation, loading and elution of the polysomes were as described by Schutz *et al.*, (1977).

2.16. *Tryptic Peptide Mapping of Immunochromatographed Translation Products*

Four samples were tryptic peptide mapped based on the method of Higgins and Spencer (1977).

To each sample (1 to 2 million cpm), 4 mg of purified spinach LSU was added. Protein was then precipitated with 5 percent trichloroacetic acid (TCA). The pelleted protein was washed twice with ethanol/ether and dried under vacuum before being dissolved in 0.5 ml formic acid and cooled on ice. 0.5 ml of performic acid, prepared by reacting 0.5 ml hydrogen peroxide with 9.6 ml of 98 percent formic acid at room temperature for 2 hours, was added. The protein plus performic acid, was left for 2 hours in the cold, care being taken to prevent condensation. The solution was then lyophilised for about 2 hours to remove the performic acid, dissolved in 1 ml formic acid and 10 ml H_2O, allowed to stand 1 hour at room temperature and re-lyophilised overnight.

For trypsin digestion the protein was dissolved in 50 mM ammonium bicarbonate, pH 8.4 to a concentration of 2 mg/ml. Trypsin (Worthington, TPCK-treated) was dissolved in water to 1 mg/ml and added to the protein solution to give 10 µg trypsin per mg protein. This was incubated at 37°C for 4 hours prior to the addition of a further 5 µg trypsin per mg protein for overnight digestion at 37°C.

After digestion the sample was lyophilized overnight then redissolved in 2 ml H_2O and 4 drops of ammonium bicarbonate (50 mM, pH 8.4) followed by 1 drop of acetic acid. Insoluble material was centrifuged down and the supernatant lyophilized overnight. The dry trypsin-digested protein was dissolved in 20 µl H_2O and applied to a sheet of Whatman 3 MM paper (37.5 x 60 cm) in a 2 cm long line. The tube was washed with 10 µl H_2O and this was also applied to the paper.

The samples were chromatographed by ascending chromatography twice for 16 hours in n-butanol:acetic acid:water:pyridine (15:3:12:10), the paper being dried between the runs.

After chromatography the samples were electrophoresed in water: pyridine:acetic acid (289:1:14). The paper was wet with the buffer so that the buffer concentrated the chromatographed line. Electrophoresis was under water cooled kerosine at 3000 volts for 75 minutes (Amperage, 100 to 125 mA). The papers were then dried and stained with 1 percent ninhydrin dissolved in a solution containing 0.1 g cadmium chloride, 10 ml water, 5 ml acetic acid and 100 ml acetone (dissolved in the order given). The stained paper was photographed and then placed in contact with X-ray film (Kodak RP 5) for one to two months autoradiography.

2.17. *The Iodination and Mapping of Chloroplast RNA Preparations onto Spinach Chloroplast DNA.*

The techniques used for the iodination and mapping of spinach chloroplast RNA were essentially as described by Whitfeld *et al.*, (1978). The RNA preparations, iodinated by the method of Getz *et al.*, (1972), were hybridised to cellulose nitrate membranes containing the restriction endonuclease fragments of spinach chloroplast DNA. The membranes were prepared by the transfer of denatured DNA fragments from agarose gels as described by Southern (1975). After hybridisation of the ^{125}I-labelled RNA to the membranes, followed by comprehensive washing and ribonuclease digestion to remove un-hybridised RNA, the membranes were placed in contact with X-ray film and the DNA bands containing complementary sequences to the RNA preparations were identified. The only differences between the procedure of Whitfeld *et al.*, and that used here was in the purification of the iodinated RNA by chromatography on lysine-sepharose 4B (Pharmacia) rather than CF-11 cellulose and in some of the reaction mixtures. The restriction

endonuclease digestion mixtures for Sma I, Pst I and Eco R1 were as given by Whitfeld *et al.*,(1978); the digestion mixture for Hind III contained 5 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 50 mM NaCl, 0.1 mg/ml BSA and 6 mM β-mercaptoethanol; for Bam I the mixture contained 5 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 50 mM NaCl, 0.1 mg/ml BSA and 8 mM β-mercaptoethanol. The hybridization reactions of ^{125}I-labelled RNA with DNA fragments on cellulose nitrate membrane strips contained 1.7 ml of formamide buffer (Whitfeld *et al.*, 1978), 2 µg of *E. coli* ribosomal RNA and 1 to 2 x 10^5 cpm of iodinated RNA.

CHAPTER 3

Protein Synthesis during Chloroplast Development.

3.1 INTRODUCTION

It was pointed out in Chapter 1 that during the greening of dark grown plants the synthesis of proteins assumes great importance. Regulation of protein synthesis can be exerted through both transcriptional and translational control mechanisms and there is evidence (Chapter 1) that both types of control operate in the nuclear-cytoplasm system of leaf cells. Is the same true of chloroplast protein synthesis? In this chapter the nature of the regulation of protein synthesis during chloroplast development will be examined. The synthesis of chloroplast proteins generally, and of the LSU of RuBPCase in particular, are analysed in terms of three control parameters: messenger RNA levels, polysome size and transit time.

The rate of synthesis of a given protein depends both upon the amount of mRNA present and upon the efficiency with which that messenger is used as a template.

The template activity will, in turn, be dependent upon the number of ribosomes associated with the messenger and upon the time required for the ribosomes to traverse the length of the messenger (transit time). Therefore, given the level of messenger RNA, the number of ribosomes that will associate with the messenger and their transit time, one should be able to determine whether transcriptional control, translational control, or both are operating during development. If translational control is involved, it should also be possible to ascertain where it exerts its effect (initiation, elongation or termination of polypeptide

synthesis) and its relative significance.

There are, however, many problems associated with measuring these parameters. Messenger RNA levels are most reliably and accurately determined by using a pure, radioactive probe to whatever messenger is being quantified (e.g., Levy and Aviv, 1976; Robbins and Schimke, 1978). The extent of hybridization of the probe to total RNA preparations from the tissue indicates the number of sequences in the preparation complementary to the probe. The purity of the probe is very important and the difficulties of obtaining purified messenger RNAs for use in the production of a radioactive probe will be described in Chapter 4. Probes may also be made to mRNA released from polysomes by EDTA or puromycin treatment or to Poly-A containing RNA but neither technique is appropriate for chloroplast mRNAs (see Chapter 4).

An alternative, and commonly used, method of messenger RNA quantitation is to measure the synthesis of specific proteins by a cell-free translation system in response to a messenger RNA preparation (Strome and Young, 1978). The products can be assessed by gel electrophoresis and autoradiography of the labelled polypeptide bands. Other techniques, such as immunoprecipitation or immuno-chromatography, peptide mapping, and 2-dimensional electrophoresis can also be used to identify and quantify the synthesis of specific polypeptides. However, whatever the technique used to identify the polypeptides made *in vitro* it is necessary to assume that the amount of protein synthesised is proportional to the amount of messenger RNA present in the RNA mixture, and also that the presence of large levels of ribosomal and transfer RNA's does not affect the efficiency of the translation assay. It must also be assumed that the cell-free translation system will not show significant

discrimination between different messenger RNA species. For each of these three assumptions exceptions have been reported in the literature. However, messenger quantitation by *in vitro* translation is generally thought to provide a useful indicator of messenger levels and in the absence of a reliable probe it is the best method available. It is the technique used here.

The number of ribosomes associated with the messenger RNA can be measured in several ways. Polysome preparations are fractionated on sucrose gradients and messenger RNA levels are assayed down the gradient. The usual method of assay is to extract the RNA from polysomes of various size classes and to translate that RNA in a cell-free translation system (Robbins and Schimke, 1978; Palmiter *et al.*, 1970). The problems with this method of quantitation have been pointed out above. Alternatively, one can assay for the amount of messenger by allowing the ribosome fractions to complete translation of their messenger RNA in the presence of radioactive amino-acids and supplementary factors. Again the proteins synthesised can be assessed after gel fractionation. This *in vitro* "run-off" method of messenger quantitation is far more reliable than the direct translation assay but it is necessary to normalise the results to allow for the different numbers of ribosomes per RNA molecule.

An additional technique for measuring the number of ribosomes associated with a particular messenger will be presented in this chapter. It is dependent upon the immunological identification of the nascent protein being synthesised.

The final parameter affecting the rate of protein synthesis, transit time, is the most difficult to measure as it is necessary to determine the incorporation of a radioactive amino-acid into protein

over a time course of a few minutes. Perhaps the most reliable technique is that of Fan and Penman (1970). As shown in the diagram (Figure 3.1) the technique measures the time required for proteins released from ribosomes to be fully labelled.

After labelling with radioactive amino-acids, the nascent chains will be completely labelled in one transit time. Each completed polypeptide chain will contain an equivalent amount of label. In the second transit time, the nascent polypeptide radioactivity will remain constant. The total polypeptides labelled in the second transit time will have twice as much label as those labelled in the first transit time. Each subsequent transit time will release the same amount of label as did the second transit. The difference between the plots of soluble labelled protein compared to total labelled protein (soluble plus ribosome bound) will equal one half the mean transit time. This can be assumed to be equivalent to the rate of polypeptide chain elongation since the time required for termination appears to be virtually negligible (Lodish and Jacobsen, 1972).

The developmental system being examined is that of the greening of dark grown pea apices. This system was chosen for two principal reasons. Firstly, there are considerable data already available about the greening of peas and of particular interest is the knowledge that some of the greatest measured increases in enzyme activities associated with greening have been obtained using these plants.

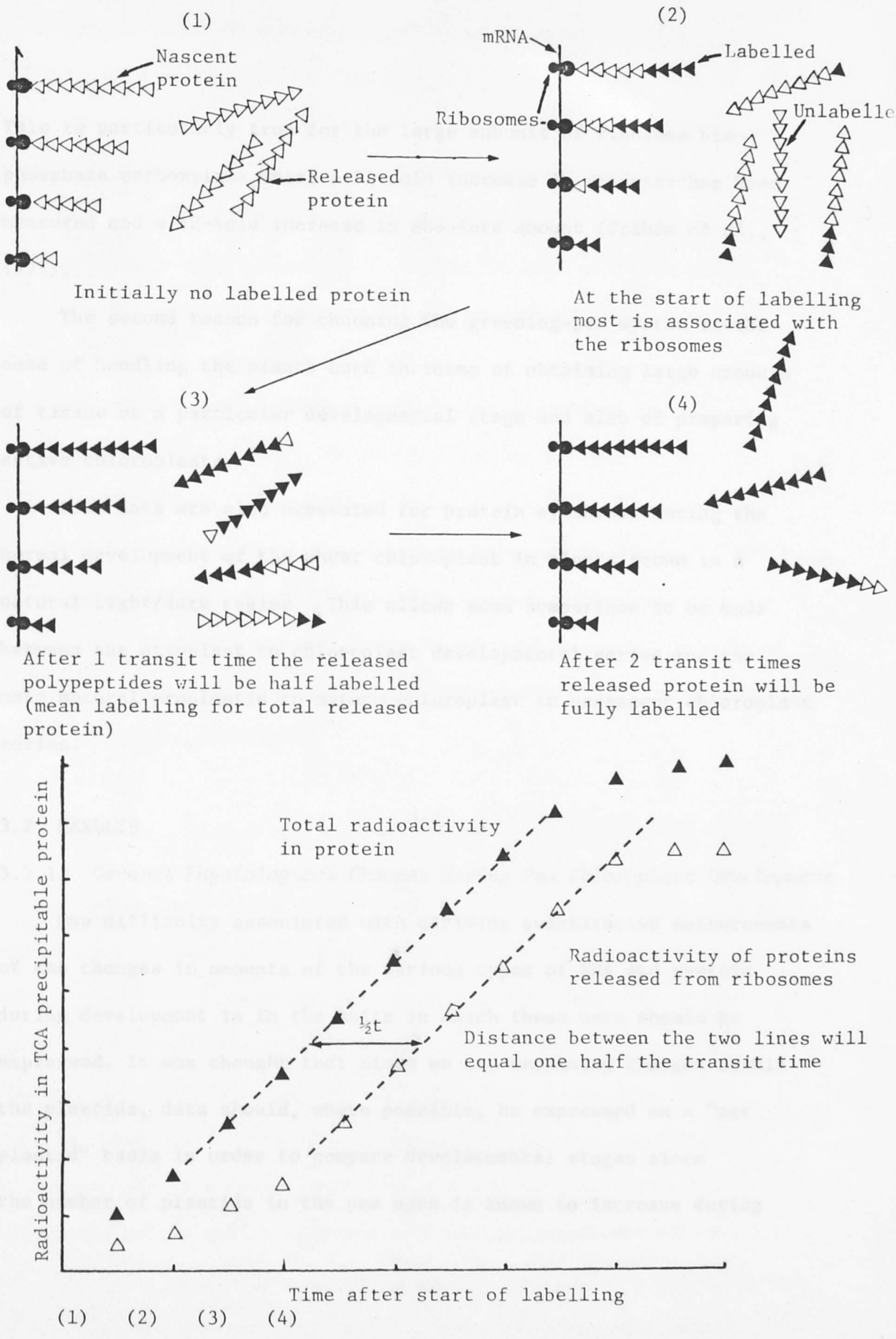


Figure 3.1. Estimation of mean ribosome transit time.

This is particularly true for the large subunit of ribulose bisphosphate carboxylase where a 91-fold increase in activity has been measured and a 12-fold increase in absolute amount (Graham *et al.*, 1971).

The second reason for choosing the greening-pea system is the ease of handling the plants both in terms of obtaining large amounts of tissue at a particular developmental stage and also of preparing active chloroplasts.

Some data are also presented for protein synthesis during the normal development of the wheat chloroplast in plants grown in a natural light/dark regime. This allows some comparison to be made between the etioplast to chloroplast developmental series and the more natural proplastid to mature chloroplast to senescent chloroplast series.

3.2 RESULTS

3.2.1. *General Physiological Changes during Pea Chloroplast Development*

One difficulty associated with deriving quantitative measurements of the changes in amounts of the various types of RNA and protein during development is in the units in which these data should be expressed. It was thought that since we are examining changes within the plastids, data should, where possible, be expressed on a "per plastid" basis in order to compare developmental stages since the number of plastids in the pea apex is known to increase during

development. Measurements will also be given on a "per apex" basis to help reveal absolute changes.

From a knowledge of the number of plastids present in the apex during development it is possible to determine the times and the extent of plastid division as the pea plants green. Knowledge of plastid numbers also allows comparison to be made with published data also providing information on the general physiological status of the tissue during greening.

(i) *Chlorophyll Synthesis and Plastid Numbers in Pea Apices*

The number of plastids in the apex can be determined from the chlorophyll content. Since chlorophyll is located entirely within plastids, a measurement of the amount of chlorophyll present in a known number of plastids (measured in a haemocytometer) and the amount of chlorophyll present in the whole apex (measured in acetone extracts by the method of Anderson and Boardman, 1964) provides the number of plastids per apex.

$$\text{number of plastids per apex} = \frac{\text{weight of chlorophyll per apex}}{\text{weight of chlorophyll per plastid}}$$

Although there is no chlorophyll in etioplasts, protochlorophyll can be used to make similar measurements (Anderson and Boardman, 1964). It was also necessary to determine what proportion of the isolated chloroplasts (used to obtain the weight of chlorophyll per plastid) as viewed through the microscope were indeed plastids. This is a particular problem with etioplasts and the early stages of chloroplast development when the plastids are seen as small refractile bodies easily confused with starch grains and mitochondria. The proportion of plastids in the etioplast preparations was estimated by fluorescence microscopy to be 70 percent (Anderson and Boardman,

1964). This percentage rose to 75 percent for the sample from plants greened for eight hours. The remaining times in the series had easily recognisable plastids and this procedure was not necessary. The pea seedlings were grown for 180 hours (after start of imbibition) in the dark prior to transfer to the light.

The amount of chlorophyll present per apex and the fresh weight of the apices are plotted in Figure 3.2. These data were used in conjunction with the information in Figure 3.3 (amount of chlorophyll per plastid) to estimate the number of plastids present in an apex during greening (also in Figure 3.3).

There is little or no plastid multiplication within the first twenty hours of greening. However, within 100 hours there has been nearly a fifty-fold increase in the number of plastids per apex. The early stages of plastid greening are marked by a two-fold increase in fresh weight within the first few hours followed by a period of little fresh weight increase until 20 hours after transfer to the light. This initial jump in fresh weight is due to the rapid uptake of water by the seedlings in response to light (Alberte *et al.*, 1972, 1973).

(ii) *Polysomes of Greening Peas*

It was mentioned in the introduction to this chapter that the pattern of polysome profiles of tissues is frequently taken as a measure of the activity of protein synthesis. The data obtained from profiles are expressed as the proportion of the ribosomes which are in the form of polysomes of two or more ribosomes. The monosome peak (80S) on the profiles is assumed to represent free or inactive ribosomes.

Figure 3.2. The chlorophyll content per apex was estimated from acetone extracts of etiolated pea apices that had been allowed to green for 0, 8, 20, 40 and 95 hours. The mean apex fresh weight was measured from 20 apices greened for the indicated times.

Figure 3.3. The amount of chlorophyll present in a plastid was calculated from acetone extracts of a known number of plastids (measured in a haemocytometer). The number of plastids per apex equals the amount of chlorophyll per apex (from Figure 3.2) divided by the amount of chlorophyll per plastid (Figure 3.3).

Figure 3·2 Pea Apex Chlorophyll Content and Fresh Weight During Greening

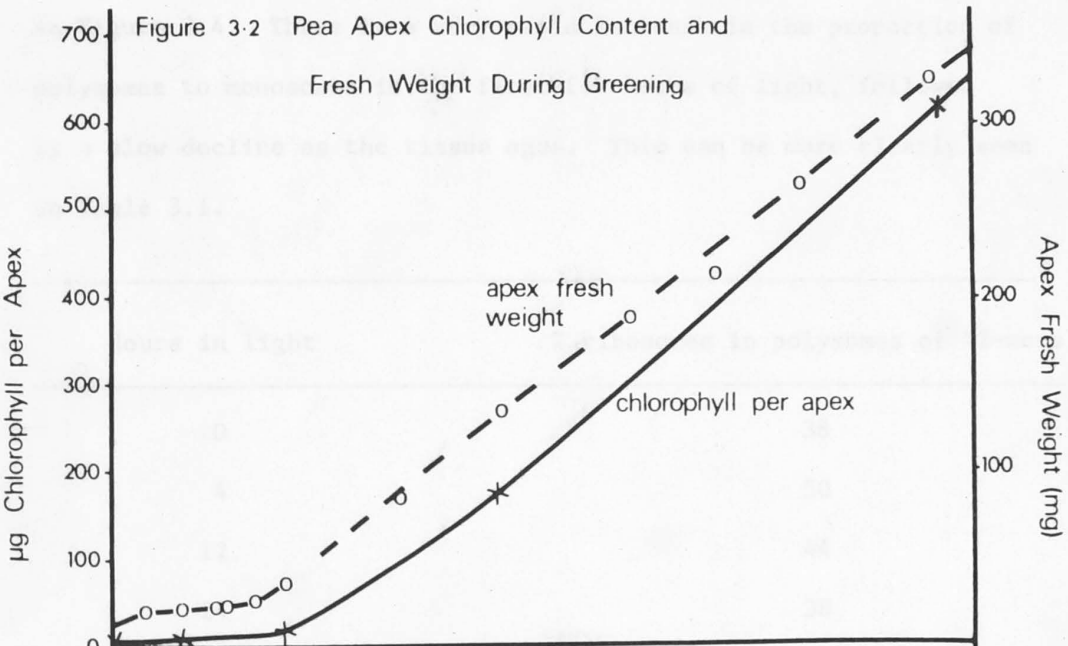
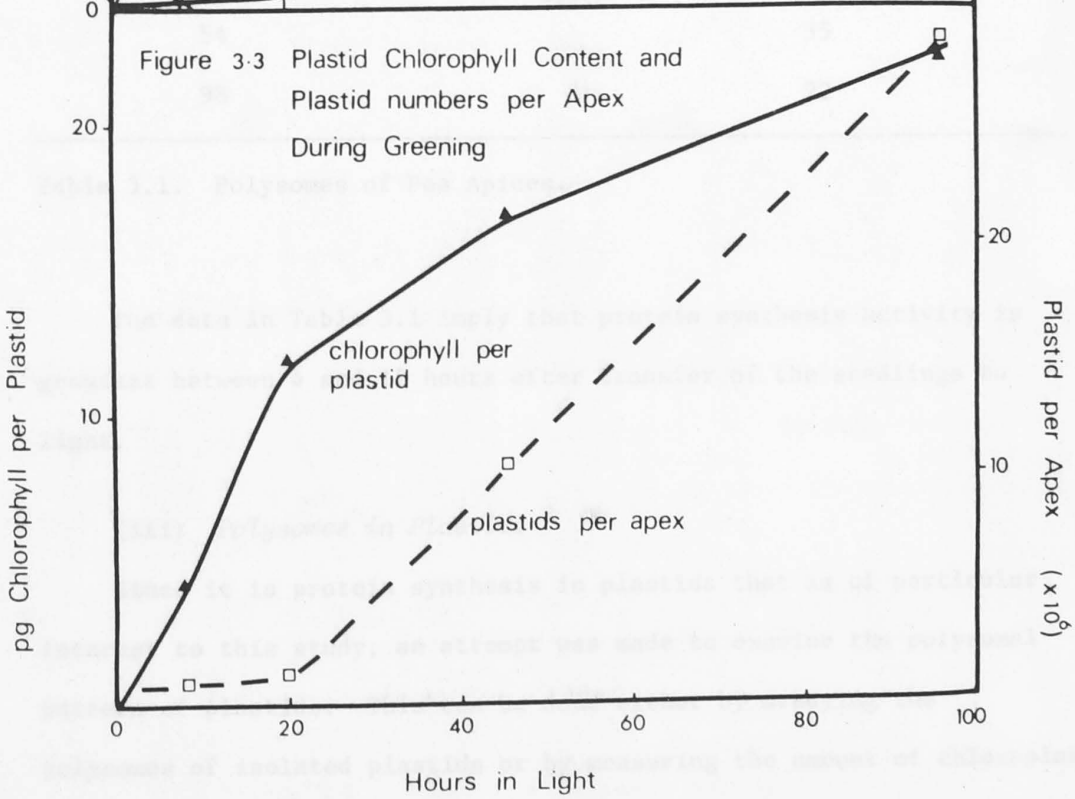


Figure 3·3 Plastid Chlorophyll Content and Plastid numbers per Apex During Greening



The total polysome profiles of the greening pea apex are displayed in Figure 3.4. There is a very rapid increase in the proportion of polysomes to monosomes in the first few hours of light, followed by a slow decline as the tissue ages. This can be more clearly seen in Table 3.1.

Hours in light	% ribosomes in polysomes of >2-mers
0	38
4	50
12	44
24	38
54	35
98	22

Table 3.1. Polysomes of Pea Apices.

The data in Table 3.1 imply that protein synthesis activity is greatest between 4 and 12 hours after transfer of the seedlings to light.

(iii) *Polysomes in Plastids*

Since it is protein synthesis in plastids that is of particular interest to this study, an attempt was made to examine the polysomal pattern of plastids. This can be done either by studying the polysomes of isolated plastids or by measuring the amount of chloroplast RNA in fractions from a sucrose gradient analysis of total apical

Figure 3.4. The plots shown in this figure represent the absorbance at 260 nm of polysomes prepared from pea apices by the method of Davies *et al.* (1972) and separated on isokinetic sucrose gradients. The 80S cytoplasmic ribosome peak is indicated on the etiolated apex polysome profile and the location of the di-, tri-, tetra-, etc. polysome peaks are also indicated (numbered 2, 3, 4, etc.).

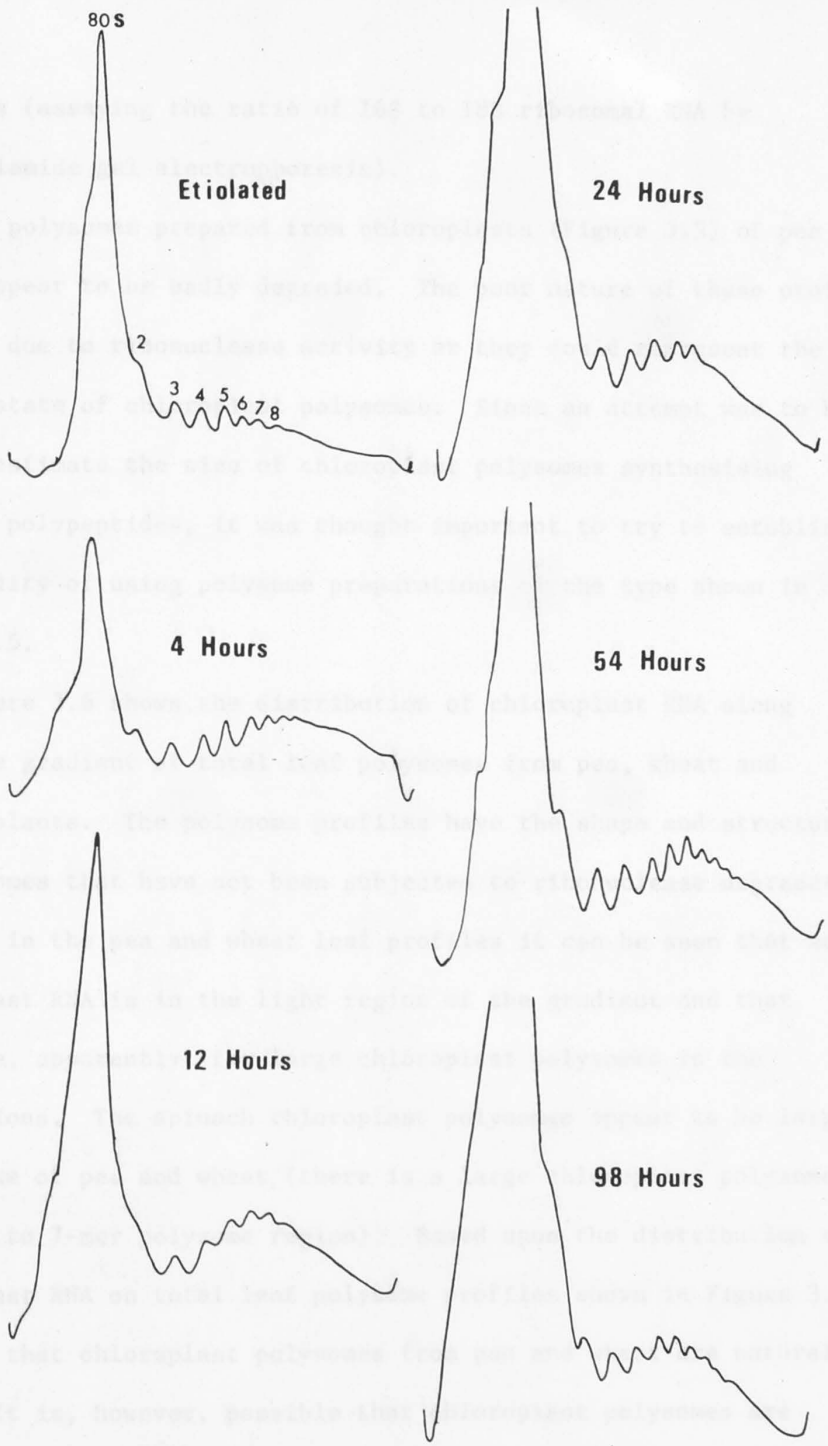


Figure 3·4 Total Pea Polysome Profiles

polysomes (assaying the ratio of 16S to 18S ribosomal RNA by polyacrylamide gel electrophoresis).

The polysomes prepared from chloroplasts (Figure 3.5) of pea apices appear to be badly degraded. The poor nature of these profiles could be due to ribonuclease activity or they could represent the natural state of chloroplast polysomes. Since an attempt was to be made to estimate the size of chloroplast polysomes synthesising specific polypeptides, it was thought important to try to establish the validity of using polysome preparations of the type shown in Figure 3.5.

Figure 3.6 shows the distribution of chloroplast RNA along a sucrose gradient of total leaf polysomes from pea, wheat and spinach plants. The polysome profiles have the shape and structure of polysomes that have not been subjected to ribonuclease degradation. However, in the pea and wheat leaf profiles it can be seen that most chloroplast RNA is in the light region of the gradient and that there are, apparently, few large chloroplast polysomes in the preparations. The spinach chloroplast polysomes appear to be larger than those of pea and wheat (there is a large chloroplast polysome peak in the 5 to 7-mer polysome region). Based upon the distribution of chloroplast RNA on total leaf polysome profiles shown in Figure 3.6, it seems that chloroplast polysomes from pea and wheat are naturally small. It is, however, possible that chloroplast polysomes are more susceptible to ribonuclease attack than the polysomes of the cytoplasm. A related question, that of the stability of chloroplast messenger RNA's, will be discussed in Chapter 4.

Figure 3.5. The polysomes displayed in this figure were prepared from isolated pea plastids and, as for Figure 3.4, the profiles represent the absorbance at 260 nm of polysomes separated on isokinetic sucrose gradients. The peaks labelled 30S, 50S, 70S and 80S were identified by recovering the RNA from the respective peaks and identifying the major species of RNA present by analytical polyacrylamide gel electrophoresis.

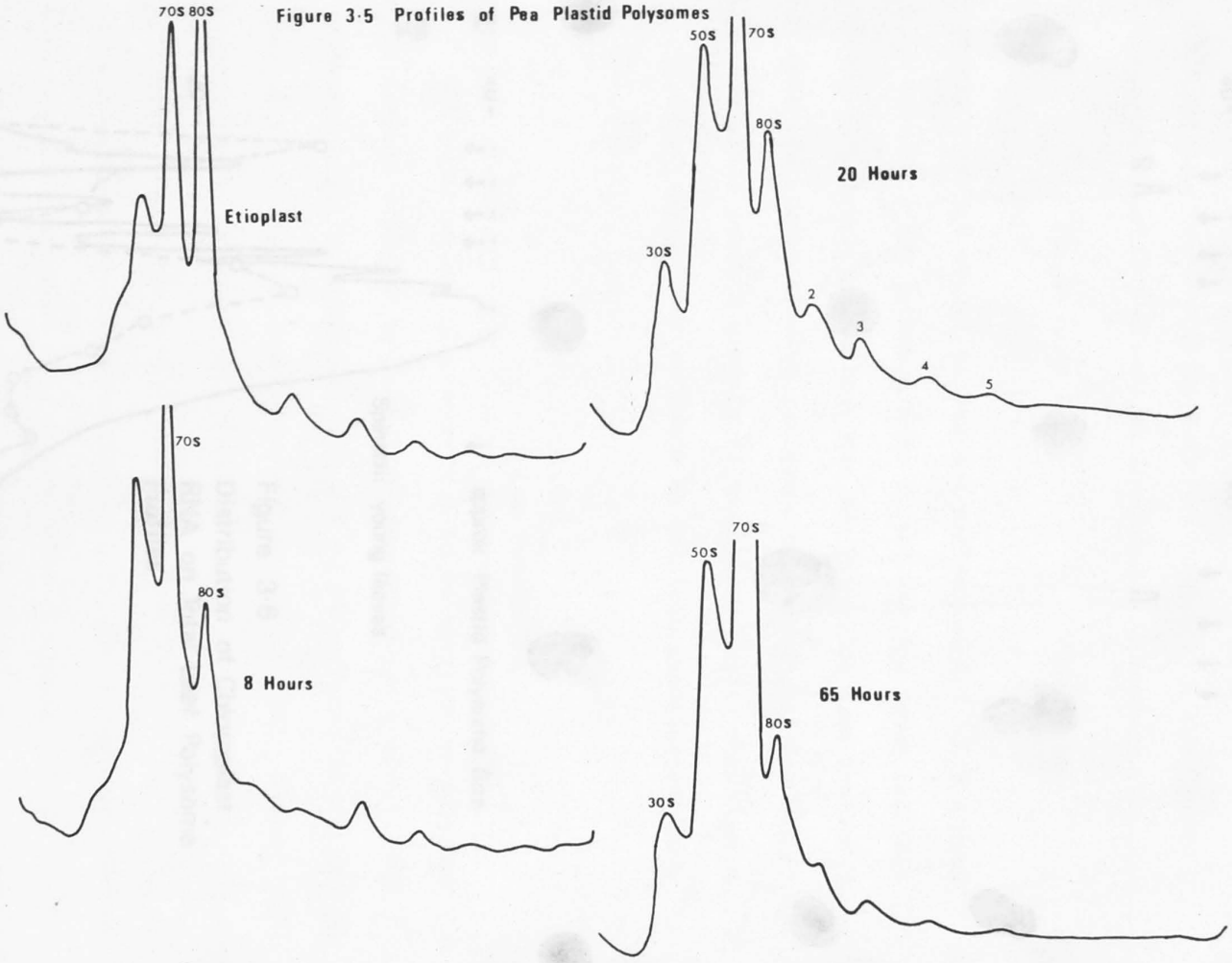


Figure 3·5 Profiles of Pea Plastid Polysomes

Figure 3.6. Polysomes from pea apices (after 24 hours greening), wheat (9 days old) and young spinach leaves were prepared and displayed as for Figure 3.4. Fractions were collected and analysed by polyacrylamide gel electrophoresis to measure the ratio of chloroplast to cytoplasmic RNA down the gradients. Using these ratios the amount of plastid RNA was calculated and plotted over the polysome profiles as shown on the figure.

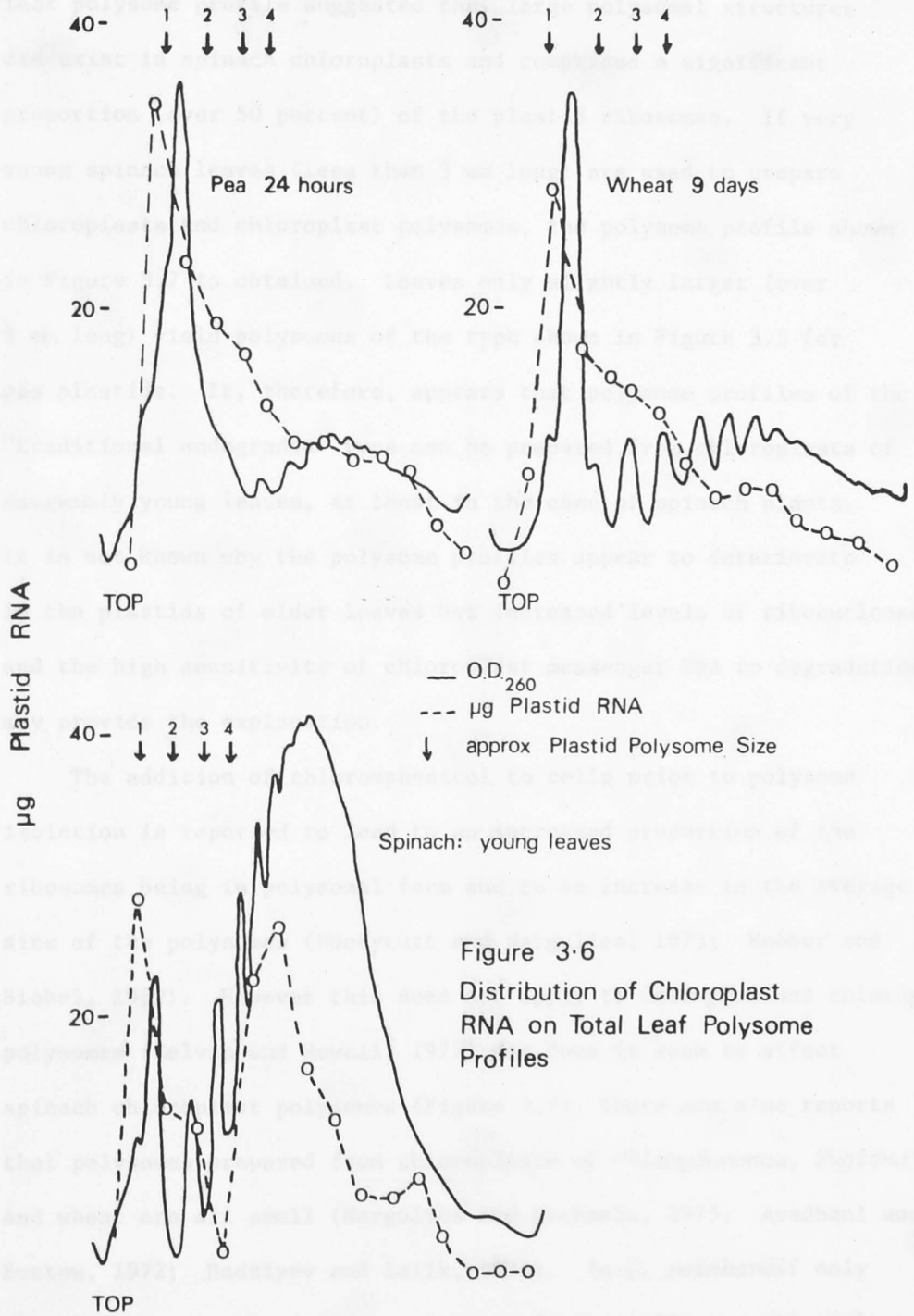


Figure 3·6
Distribution of Chloroplast RNA on Total Leaf Polysome Profiles

The distribution of chloroplast RNA on the total spinach leaf polysome profile suggested that large polysomal structures did exist in spinach chloroplasts and comprised a significant proportion (over 50 percent) of the plastid ribosomes. If very young spinach leaves (less than 3 mm long) are used to prepare chloroplasts and chloroplast polysomes, the polysome profile shown in Figure 3.7 is obtained. Leaves only slightly larger (over 5 mm long) yield polysomes of the type shown in Figure 3.5 for pea plastids. It, therefore, appears that polysome profiles of the "traditional undegraded" type can be prepared from chloroplasts of extremely young leaves, at least in the case of spinach plants. It is not known why the polysome profiles appear to deteriorate in the plastids of older leaves but increased levels of ribonuclease and the high sensitivity of chloroplast messenger RNA to degradation may provide the explanation.

The addition of chloramphenicol to cells prior to polysome isolation is reported to lead to an increased proportion of the ribosomes being in polysomal form and to an increase in the average size of the polysomes (Honeycutt and Margulies, 1973; Hoober and Blobel, 1969). However this does not apply to *Chlamydomonas* chloroplast polysomes (Gelvin and Howell, 1977) nor does it seem to affect spinach chloroplast polysomes (Figure 3.8). There are also reports that polysomes prepared from chloroplasts of *Chlamydomonas*, *Euglena* and wheat are all small (Margulies and Michaels, 1975; Avadhani and Buetow, 1972; Hadziyev and Zalik, 1970). In *C. reinhardii* only about eight percent of large polysomes in the cells are chloroplast derived, whereas 25 percent of small polysomes are from plastids (Baumgartel and Howell, 1976).

Figure 3.7. The polysomes displayed were prepared from isolated spinach chloroplasts. The location of the 70S (monosomes), di-, tri- and tetra-somes are indicated.

Figure 3.9. Membrane polysomes were isolated from plastid membrane pellets produced by the lysis of plastids in hypotonic buffer and centrifugation at 3,000 x g. After dissolving the membranes in 5% Triton X-100 the membrane-associated polysomes were collected by centrifugation. Free polysomes were collected by high speed centrifugation of the supernatant fraction produced by lysis of the plastids and the washings from the plastid membranes.

Figure 3.7. Spinach chloroplast polysomes from very young leaves (<3 mm long)

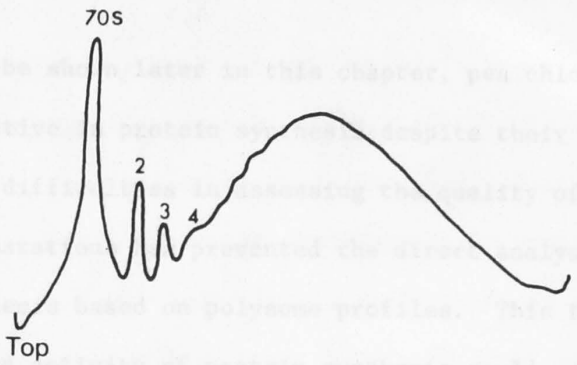
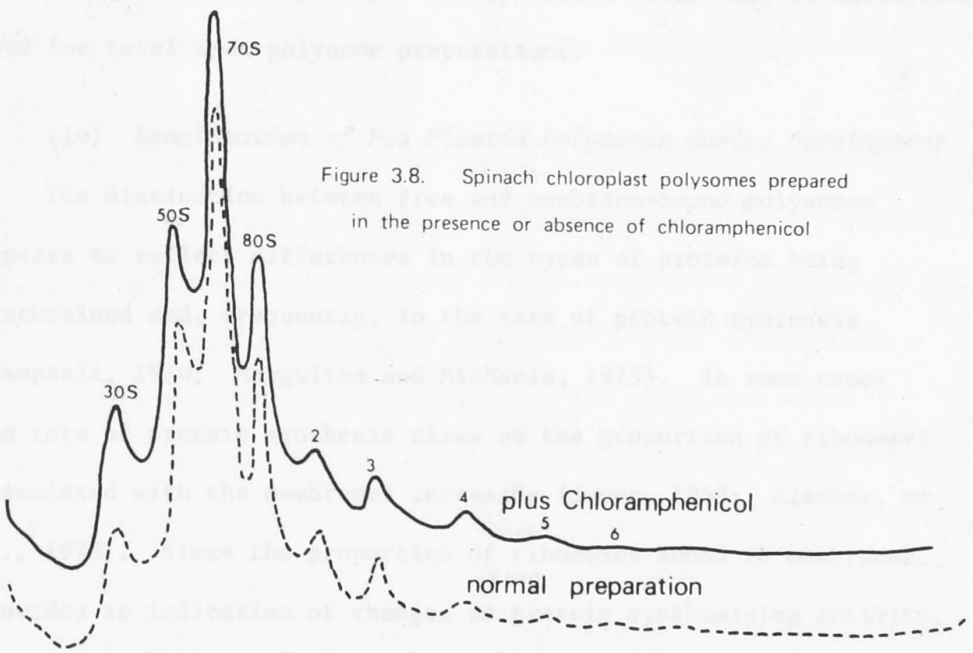


Figure 3.8. Spinach chloroplast polysomes prepared in the presence or absence of chloramphenicol



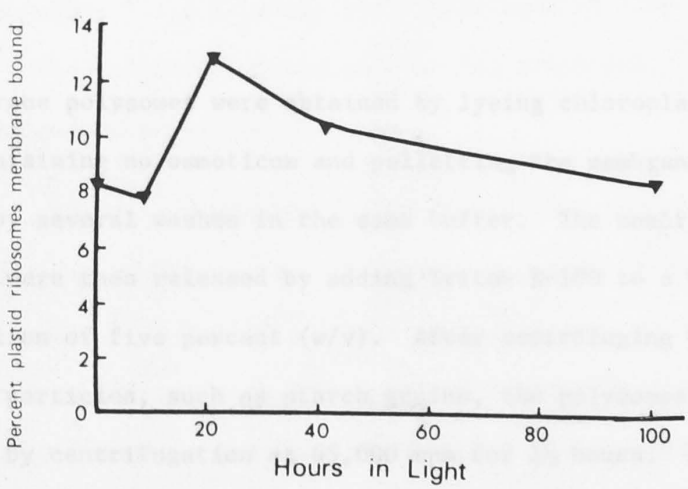


Figure 3.9. Proportion of plastid polysomes bound to membranes

As will be shown later in this chapter, pea chloroplast polysomes are highly active in protein synthesis despite their small size. However, the difficulties in assessing the quality of plastid polysome preparations has prevented the direct analysis of plastid protein synthesis based on polysome profiles. This technique of estimating the activity of protein synthesis could only be satisfactorily used for total apex polysome preparations.

(iv) *Localization of Pea Plastid Polysomes during Development*

The distinction between free and membrane-bound polysomes appears to reflect differences in the types of proteins being synthesised and, frequently, in the rate of protein synthesis (Campbell, 1970; Margulies and Michaels, 1975). In some cases the rate of protein synthesis rises as the proportion of ribosomes associated with the membranes increases (Jones, 1969; Alscher, *et al.*, 1978). Since the proportion of ribosomes bound to membranes provides an indication of changes in protein synthesising activity, this proportion was measured within plastids during chloroplast formation.

Membrane polysomes were obtained by lysing chloroplasts in buffer containing no osmoticum and pelletting the membranes at 3,000 g, followed by several washes in the same buffer. The membrane-bound polysomes were then released by adding Triton X-100 to a final concentration of five percent (w/v). After centrifuging out large insoluble particles, such as starch grains, the polysomes were recovered by centrifugation at 65,000 rpm for 1½ hours. Free polysomes were prepared from the supernatant fractions of the

membrane washings. Most of the chloroplast's protein synthesis is concerned with the production of the LSU protein (a soluble protein) which occurs on free polysomes (see next section). The proportion of ribosomes bound to the chloroplast membranes might, therefore, be expected to be low. That this is so is shown in Figure 3.9, where the percentage of ribosomes associated with membranes has been plotted against the time the pea seedlings have been exposed to light.

The fluctuations in proportion of membrane-bound polysomes to free polysomes during development are not large. They do, however, appear to follow the quantitative changes that occur in plastid protein synthesis over the same developmental time (see later).

The values presented in Figure 3.9 are lower than those reported elsewhere. Tao and Jagendorf (1973) obtained percentages of between 18 and 20 for pea and spinach thylakoid-bound polysomes. Even higher amounts, over 50 percent, were claimed for tobacco and *C. reinhardii* bound polysomes (Chen and Wildman, 1970; Chua *et al.*, 1973). However, it is possible that these workers lost some of the plastids' free ribosomes during chloroplast preparation as suggested by Tao and Jagendorf (1973).

3.2.2. *Protein Synthesis during Pea Plastid Development*

Protein synthesis during chloroplast development can be observed in several ways. Firstly, by examining the amounts of protein being accumulated in chloroplasts over a period of pea plastid development. Secondly, by measuring the ability of isolated pea plastids to incorporate radioactive amino-acids into proteins. Thirdly, by following the incorporation of labelled amino-acids into intact

plants or apices.

(i) *Protein Accumulation during Pea Plastid Development.*

Proteins extracted from the plastids of pea apices at various times after transfer to the light can be fractionated by SDS-polyacrylamide gel electrophoresis. An indication of the amount of total protein present as well as that for particular polypeptides can be assessed by staining with Coomassie Blue. Although the ability of different proteins to take up this dye may vary, it is a safe assumption that, for a given protein, staining provides a valid comparison of changes in relative amounts as development proceeds. Figure 3.10 (a and b) shows the stained pattern and a diagrammatic interpretation of the pattern for two gradient gels; 3.10 (a) shows the soluble proteins released when chloroplasts are lysed in a hypotonic buffer and 3.10 (b) shows the membrane proteins that remain associated with the membranes after four washes with hypotonic buffer. Both gels were loaded so that each channel contained the proteins present in approximately 7,000 plastids. The channels, from left to right, show the proteins from 0, 8, 20, 40 and 100 hour greened pea plastids (the "hours in light" under the diagrams are on a log scale).

Amongst the soluble proteins the large and small subunits of RuBPCase are by far the most abundant. Both these subunits only begin to accumulate after about 20 hours in the light. Other proteins can be seen to increase in amount during chloroplast development (e.g., at 45, 34 and 20 thousand daltons). There are also several polypeptides that are present only in the etioplasts and immature chloroplasts (e.g., at 58, 57, 46 and 23 thousand daltons) and a third class of proteins that appears to remain unchanged with time of development (e.g., 76, 62, 33 and 20 thousand daltons).

Figure 3.10. Approximately 7,000 plastids from each of the pea developmental times (0, 8, 20, 40 or 100 hour greened apices) were lysed in hypotonic buffer. After centrifugation the supernatant fraction yielded the soluble plastid proteins and the pellet yielded the membrane associated proteins. The proteins were separated by SDS-polyacrylamide gradient gel electrophoresis to give the coomassie stained patterns shown in the figure. Line drawings are also presented to emphasise changes that occur during plastid development (the numbers below each drawing give the developmental stage on a log scale). The approximate molecular weights of some of the major proteins are given on the right hand side of the line drawings.

Figure 3.10. Pea plastid proteins.
(Coomassie stained)

The changes that occur in the accumulation of membrane proteins are far stronger than those shown by soluble plastid proteins. Most membrane proteins only appear late in chloroplast development and are totally absent from the etioplasts (e.g., at 40, 35, 30, 26 and 24 thousand daltons, Figure 3.10b). Only three membrane proteins can be clearly identified in the etioplasts (66, 58 and 37 thousand daltons) and of these, two have apparently disappeared from the plastid membranes after 20 hours of greening.

It is possible that those proteins that show a decline in amount during development are contaminants. As pointed out earlier, the etioplast preparation is made up of only 70 percent etioplasts, the remaining refractile bodies seen by phase contrast microscopy perhaps being starch grains or mitochondria. The proportion of such contamination declines as the chloroplasts mature and become larger and easier to prepare. Contamination, therefore, is expected to be a problem only early in the developmental series.

(ii) *Incorporation of ^{35}S-Methionine into Proteins by Isolated Pea Plastids.*

Two energy sources can be used to drive protein synthesis in isolated plastids; light or ATP (Bottomley *et al.*, 1974). The pattern of incorporation of labelled methionine by pea plastids as greening progresses can be seen from the graphs in Figure 3.11. However, inability of etioplasts and very immature chloroplasts to utilise light as an energy source (due to lack of chlorophyll and photosynthetic competence) made it necessary to use the ATP-driven system in all subsequent experiments.

Both graphs in Figure 3.11 show maximum rates of incorporation at 20 to 30 hours after transfer of the plants to the light and then a decline as the chloroplasts begin to age. By one hundred hours

Figure 3.11. Isolated pea plastids were incubated for 20 minutes in the presence of ^{35}S-methionine and the incorporation of label into TCA precipitable protein was measured. The two plots given show the ability of the developing pea plastids to incorporate ^{35}S-methionine into protein using two different energy sources, light (open triangles) and ATP (closed circles). In the absence of both energy sources there is no incorporation of radioactivity.

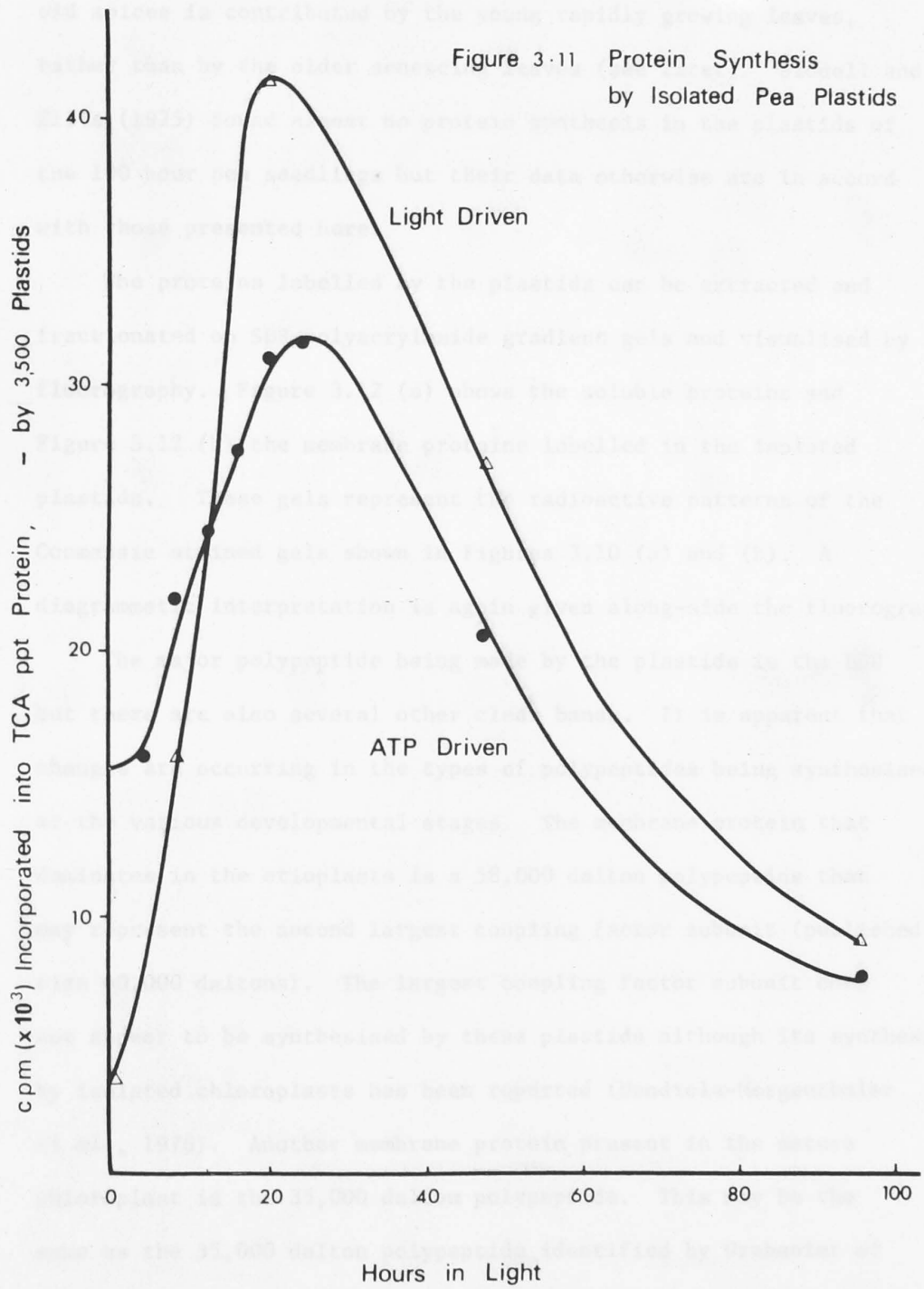


Figure 3·11 Protein Synthesis by Isolated Pea Plastids

the plastids have a rate of protein synthesis only 20 to 25 percent that of the most active plastids. Most of the synthesis in these old apices is contributed by the young rapidly growing leaves, rather than by the older senescing leaves (see later). Siddell and Ellis (1975) found almost no protein synthesis in the plastids of the 100 hour pea seedlings but their data otherwise are in accord with those presented here.

The proteins labelled by the plastids can be extracted and fractionated on SDS-polyacrylamide gradient gels and visualised by fluorography. Figure 3.12 (a) shows the soluble proteins and Figure 3.12 (b) the membrane proteins labelled in the isolated plastids. These gels represent the radioactive patterns of the Coomassie stained gels shown in Figures 3.10 (a) and (b). A diagrammatic interpretation is again given along-side the fluorographs.

The major polypeptide being made by the plastids is the LSU but there are also several other clear bands. It is apparent that changes are occurring in the types of polypeptides being synthesised at the various developmental stages. The membrane protein that dominates in the etioplasts is a 58,000 dalton polypeptide that may represent the second largest coupling factor subunit (published size 60,000 daltons). The largest coupling factor subunit does not appear to be synthesised by these plastids although its synthesis by isolated chloroplasts has been reported (Mendiola-Morgenthaler *et al.*, 1976). Another membrane protein present in the mature chloroplast is the 35,000 dalton polypeptide. This may be the same as the 35,000 dalton polypeptide identified by Grebanier *et al.*, (1978) as the precursor of the 32,000 dalton thylakoid protein.

A closer examination of the total proteins made during the first day of illumination (Figure 3.13) shows that there are

Figure 3.12. The gel patterns given in this figure show the ^{35}S-methionine labelled proteins synthesized during a 20 minute incubation period by pea plastids greened for 0 to 100 hours (as indicated). As in Figure 3.10 the proteins were separated into soluble and membrane associated. The line drawings illustrate the changes that occur in proteins synthesized during pea plastid development, the numbers below each drawing indicate the plastid developmental stage and the numbers on the right hand side, the polypeptide size.

Figure 3.12. Proteins synthesised by isolated pea plastids.
(Fluorographs)

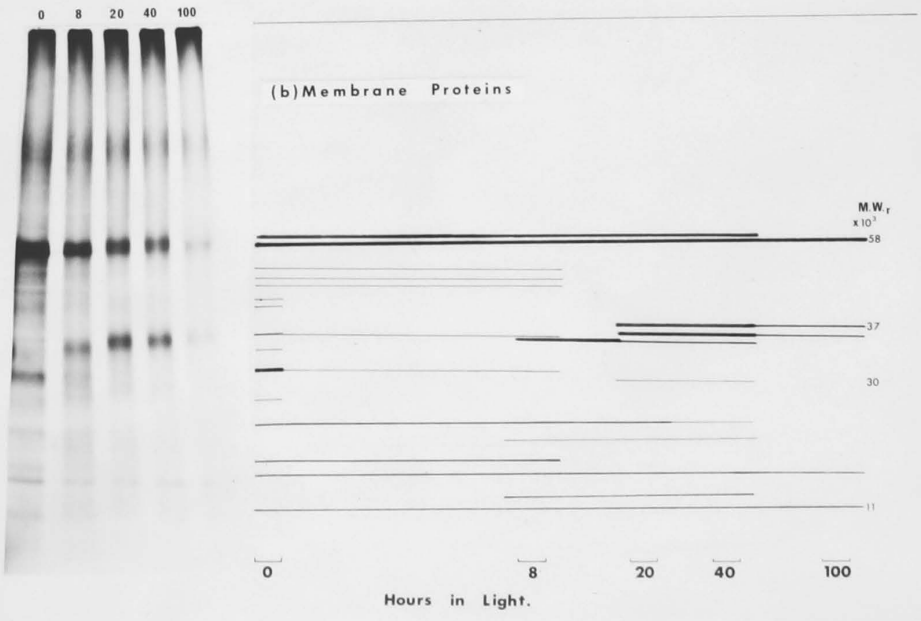


Figure 3.13. The labelled proteins in this figure were synthesized in 20 minutes by pea plastids allowed to green for 0 to 24 hours (as indicated).

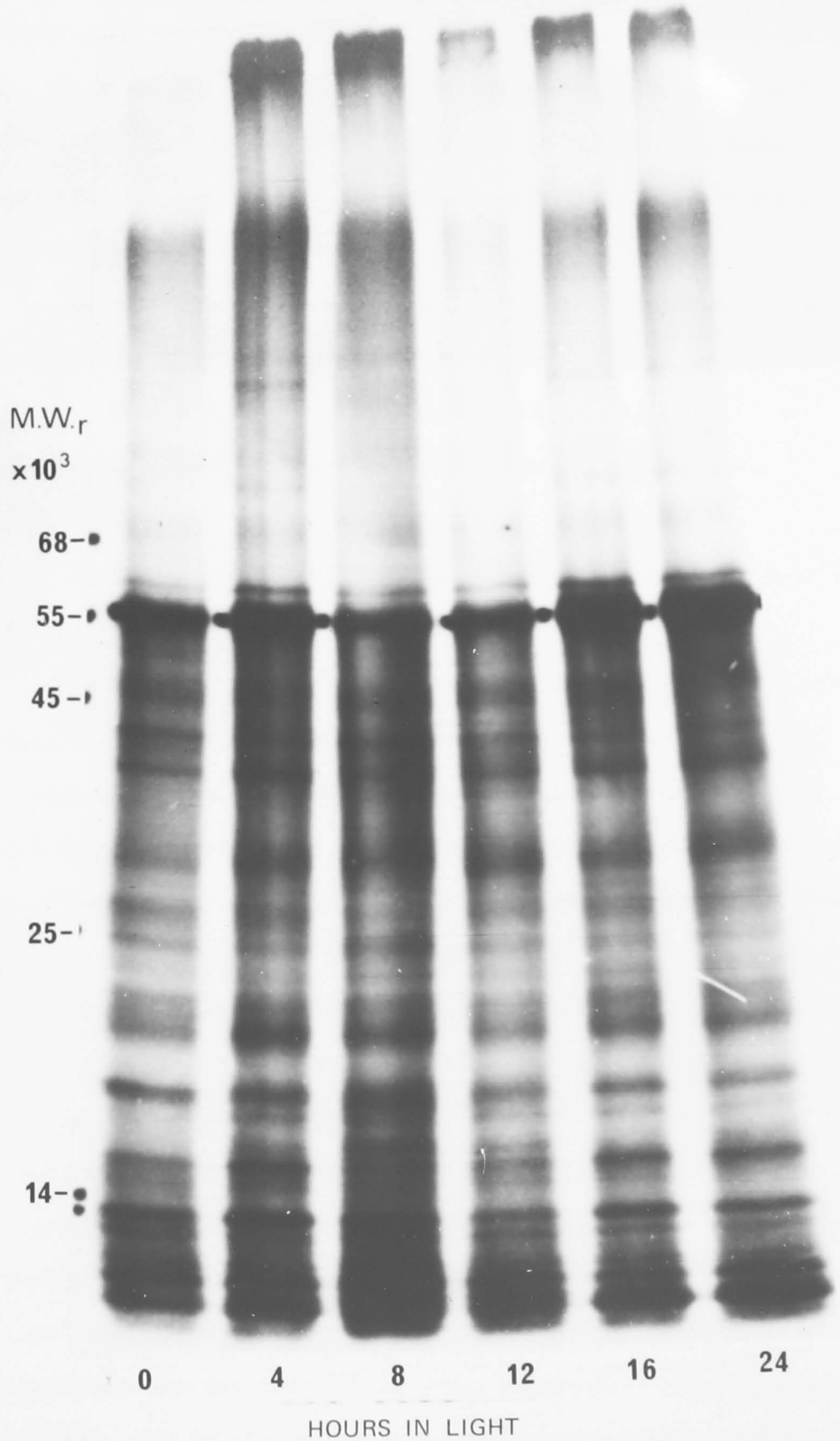


Figure 3.13. ^{35}S-methionine labelled proteins made by isolated pea plastids (Fluorograph of gradient gel)

relatively few changes in the types of polypeptides being synthesised by the plastids. However, some of these changes are apparent within the first four hours. The fluorograph in Figure 3.13 shows total plastid labelled protein loaded onto gels so that there are about equal counts in each channel.

It seems that the change in the nature of proteins being synthesised by the plastids can occur very rapidly in response to light; within four hours. However, some proteins, such as the LSU, require more than 20 hours of illumination for a full stimulation of their synthesis.

(iii) *Comparison of Techniques for the Radioactive Labelling of Pea Plastid Proteins.*

In order to establish that the data obtained using isolated plastids give a representative picture of changes that occur within the plant, the proteins of intact pea apices were labelled at two developmental stages, dark grown and after 20 hours' illumination.

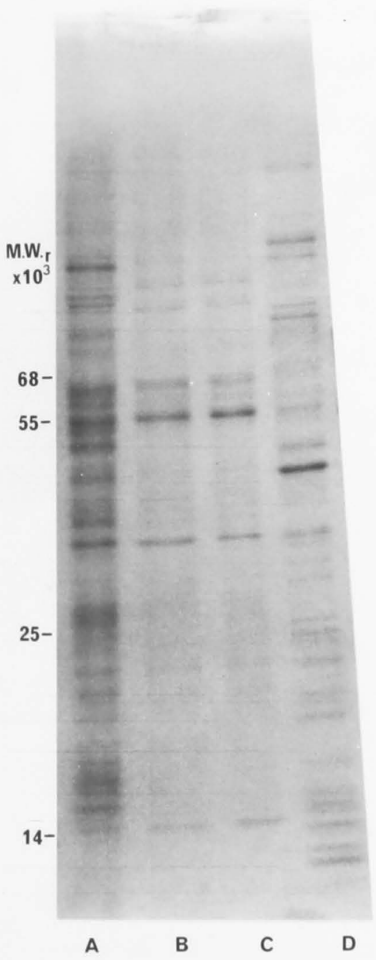
Figure 3.14 shows the Coomassie-stained pattern of an SDS-polyacrylamide gradient gel of etioplast proteins. It is clear that the fractionation of the apical proteins into total and plastid eliminates most of the stained bands. As would be expected, the pattern of labelling (Figure 3.15) is quite different in the two preparations (*in vivo* cf. *in vitro*). We can assume that the plastid polypeptides labelled in the apices but not in the isolated plastid system are plastid proteins synthesised in the cytoplasm. There are also several polypeptides that are labelled in the isolated plastid system but are not labelled *in vivo*. These could well represent proteins made by the etioplast but dependent on some cytoplasmic factors for processing into their final form. The possible lack of processing factors was suggested by Grebanier *et al.*,

Figure 3.14.

Channel	Coomassie stained proteins
A	Total etiolated apex protein
B	Etioplast protein
C	Etioplast protein from the *in organelle* system.
D	*E. coli* proteins from the *in vitro* translation system.

Figure 3.15.

Channel		
A.	Total etiolated apex protein	) Proteins labelled *in vivo*
B	Etioplast protein	)
C	Proteins labelled by isolated etioplasts (*in organelle*)	
D	Proteins synthesized by the *E. coli in vitro* translation system using pea etioplast RNA as template.	



A Total] *in vivo*
B Plastid]
C *in organelle*
D *in vitro*

Figure 3.14. Comparison of the proteins of pea etioplast labelling techniques (Coomassie stained gradient gel)

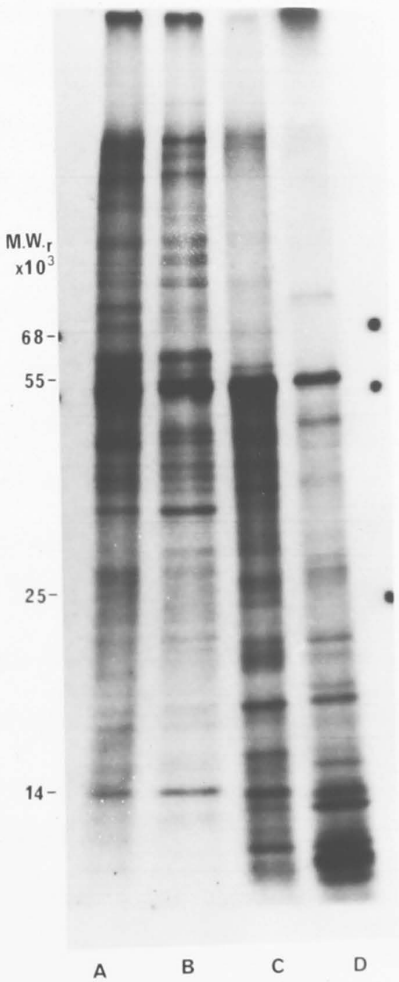


Figure 3.15. ^{35}S-methionine labelled pea proteins from etiolated apices. Comparison of labelling techniques (Fluorograph of gradient gel)

(1978) to explain discrepancies between membrane proteins synthesised by isolated maize chloroplasts and the stained bands on the same gels.

Many differences also exist between the products made when etioplast RNA is translated in an *E. coli* cell-free system and the *in vivo* and *in organelle* products. One such difference, is the synthesis of a form of the LSU of RuBPCase which has an apparently higher molecular weight than the mature LSU. This will be discussed in detail in the next chapter. The other differences between the *in vitro* and *in organelle* synthesized proteins could be due to an inability of certain messengers to be translated by the *E. coli* system, a failure in correct processing of the proteins or a premature termination of some polypeptides by the bacterial translation system. However, despite the obvious differences, the proteins synthesised *in vitro* and by isolated plastids have far more features in common than they have with the *in vivo* labelled proteins (apices).

The deductions made for the etiolated pea labelling studies just described are equally true for the pea apices after twenty hours of light. Figure 3.16 shows the fluorograph of a 20 hour pea labelling study. Again there are clear differences between the three methods for labelling chloroplast proteins as well as many similarities.

Calculations of the relative ability of the three labelling methods to incorporate label into proteins are presented in Table 3.2.

Figure 3.16.

Channel

Total)
) *in vivo* labelled protein; 60 minute labelling time
Plastid)

in organelle - isolated plastids incubated for 20 minutes with ATP as the energy source.

in vitro - 10 µg of 20 hour pea plastid RNA per 50 µl *E. coli* S-30 reaction mixture.

Figure 3.17. Detached pea apices greened for 20 hours were incubated for 30, 60 or 240 minutes in ^{35}S-methionine. The proteins shown were prepared from chloroplasts isolated from the labelled apices.

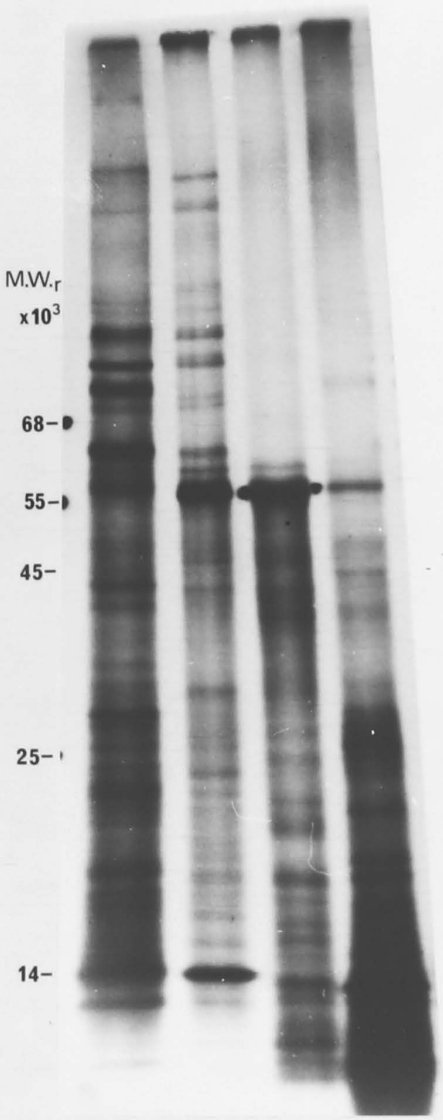


Figure 3.16. ^{35}S-methionine labelled pea proteins from apices given 20 hours illumination. Comparison of labelling techniques (Fluorograph of gradient gel)

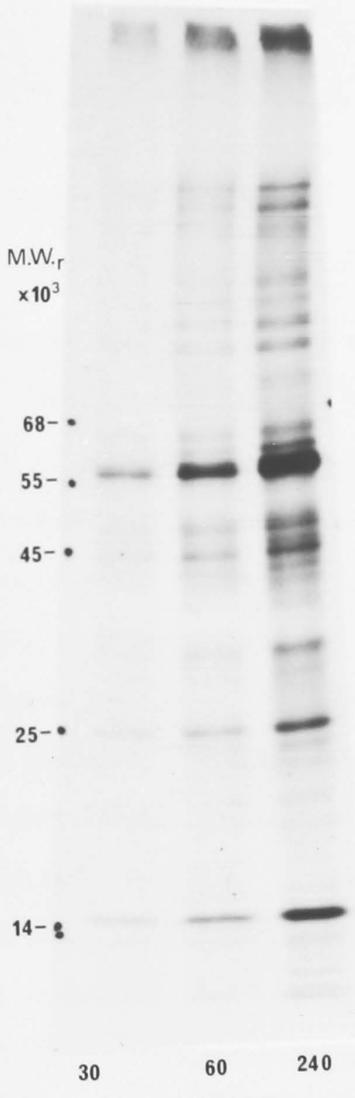


Figure 3.17. Time series of labelling of pea plastid proteins *in vivo* (Fluorograph of gradient gel)

Labelling technique	cpm/10^6 plastids		% of *in vivo*	
hours in light	0	20	0	20
in vivo (30 mins labelling)	4.07×10^6	8.98×10^6	100	100
in organelle (30 mins labelling)	3.43×10^6	7.14×10^6	84.28	79.51
in vitro (RNA from 10^6 plastids)	3.5×10^3	3.2×10^3	0.09	0.04

Table 3.2. Relative efficiency of techniques for labelling plastid proteins.

The incorporation of ^{35}S-methionine into isolated plastids compares well with the incorporation *in vivo*. This is particularly true since there is considerable label incorporated into chloroplast proteins by the cytoplasm in the *in vivo* assay. These cytoplasm-derived polypeptides could not be labelled by the isolated plastids. Continued labelling *in vivo* for more than 30 minutes, will greatly increase the specific activity of the plastid protein. Figure 3.17 shows the labelling of pea plastid protein *in vivo* after 20 hours of illumination over a four hour labelling period. It is clear that labelling is continuing throughout the four hours of the experiment and also that there is little or no change in the pattern of proteins being made, implying no gross protein turnover over these times. *In vivo* incorporation is highly efficient, over 80 percent of the labelled methionine that enters the plastids being incorporated into protein. This is consistent with published data demonstrating that pools of methionine in pea leaves are small (Bauer *et al.*, 1977).

By contrast, the incorporation of label by isolated plastids only continues for the first twenty to thirty minutes of incubation. Indeed, protein synthesis is most active for only ten to fifteen minutes and only a total of 30 to 40 percent of the counts in the

incubation medium are incorporated into protein.

Despite the differences in the pattern of labelled products from the *in vivo* compared to the *in organelle* treatment, many of the major radioactive polypeptides are identifiable in each case. The similarities between the specific activity and the labelling of the major proteins suggest that the synthesis of proteins by isolated plastids provides a useful marker of *in vivo* changes in protein synthesis. This can be seen more clearly by comparing the banding patterns for the various labelling techniques of the etioplast and 20 hour illuminated plastids.

(iv) *Protein Synthesis by Pea Plastid Polysomes.*

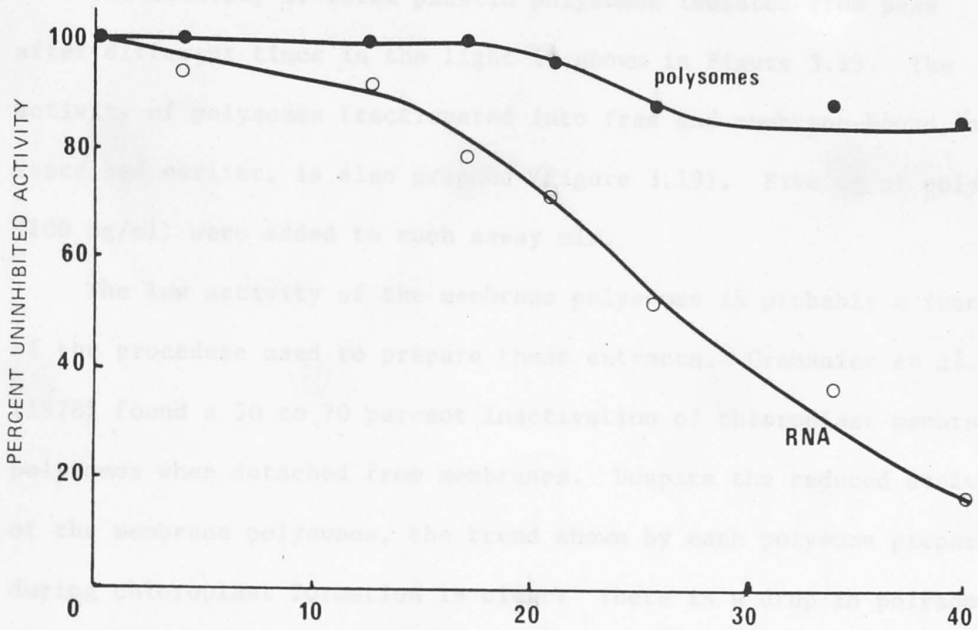
Isolated plastid polysomes can also be used to incorporate labelled amino-acids into polypeptides. The *E. coli* cell-free translation system was used to provide the necessary ingredients for pea plastid polysomes to complete translation of the mRNA by running their ribosomes off the messenger RNA molecule and releasing radioactively labelled polypeptides. The products of these "run-off" assays can be fractionated on SDS-polyacrylamide gels and visualised by fluorography.

In the assay system used, only about 10 percent of the incorporation of ^{35}S-methionine into polypeptides was due to the initiation of new protein synthesis. This observation is based on the fact that aurin-tricarboxylic acid (ATA), which at low concentrations prevents ribosomes from attaching to mRNA (Stewart, 1973), inhibited the polysome directed protein synthesis by only 10 percent (Figure 3.18). The same concentration of inhibitor caused over 80 percent inhibition of protein synthesis in the *E. coli* cell-free system when plastid RNA was used as a template (Figure 3.18). The failure of ATA to inhibit the activity of the polysome preparations to any great extent is indicative of the absence of appreciable amounts of messenger ribo-

Figure 3.18. 10 µg of chloroplast RNA or chloroplast polysomes were used as template for the *E. coli* S-30 translation system in the presence of various concentrations of ATA (total reaction volume 50 µl). The incorporation of radioactivity into TCA precipitable protein was estimated and is given as a percentage of the control (uninhibited) assay.

Figure 3.19. The ability of pea plastid polysomes to incorporate ^{35}S-methionine into TCA precipitable protein was measured by incubating 20 µg of polysomes per 50 µl *E. coli* S-30 translation assay in the presence of 40 µM ATA. Membrane and free polysomes were prepared from isolated plastids lysed in hypotonic buffer, the soluble fraction yielding the free polysomes and the pelleted membrane fraction yielding the membrane-associated polysomes.

Figure 3.18. Inhibition of *in vitro* protein synthesis by Aurin Tricarboxylic acid (ATA)



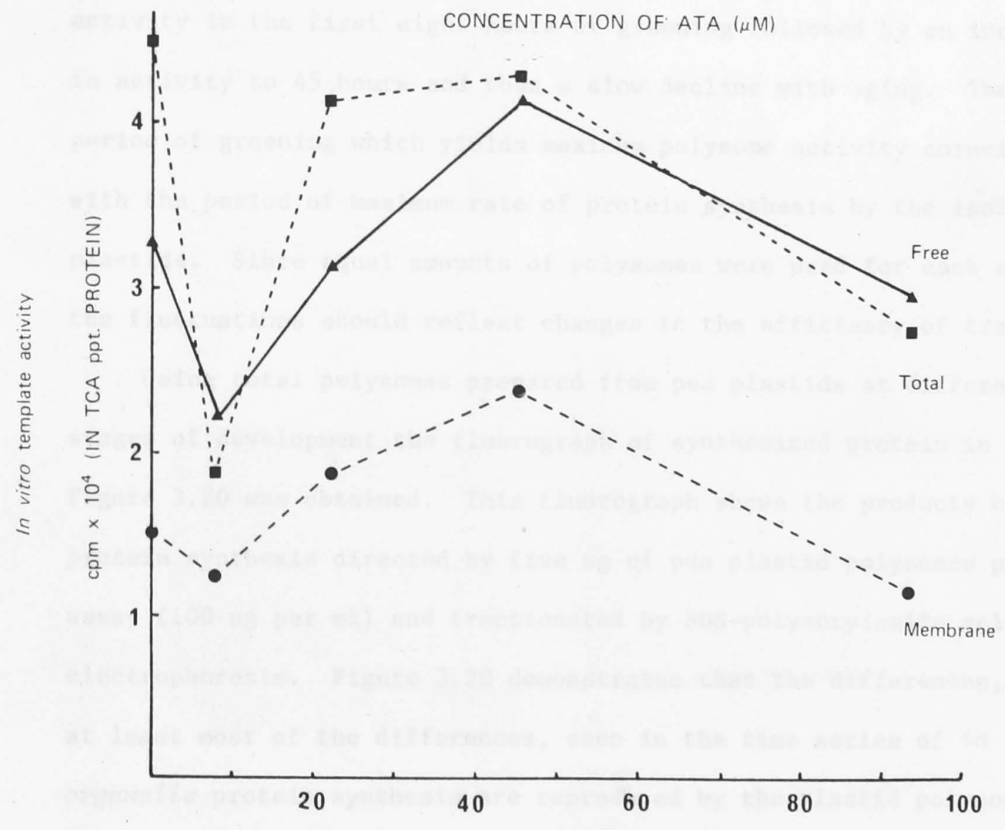


Figure 3.19. *In vitro* activity of free, membrane-bound and total plastid polysomes from greening peas.

nucleoprotein (mRNP) in the preparations.

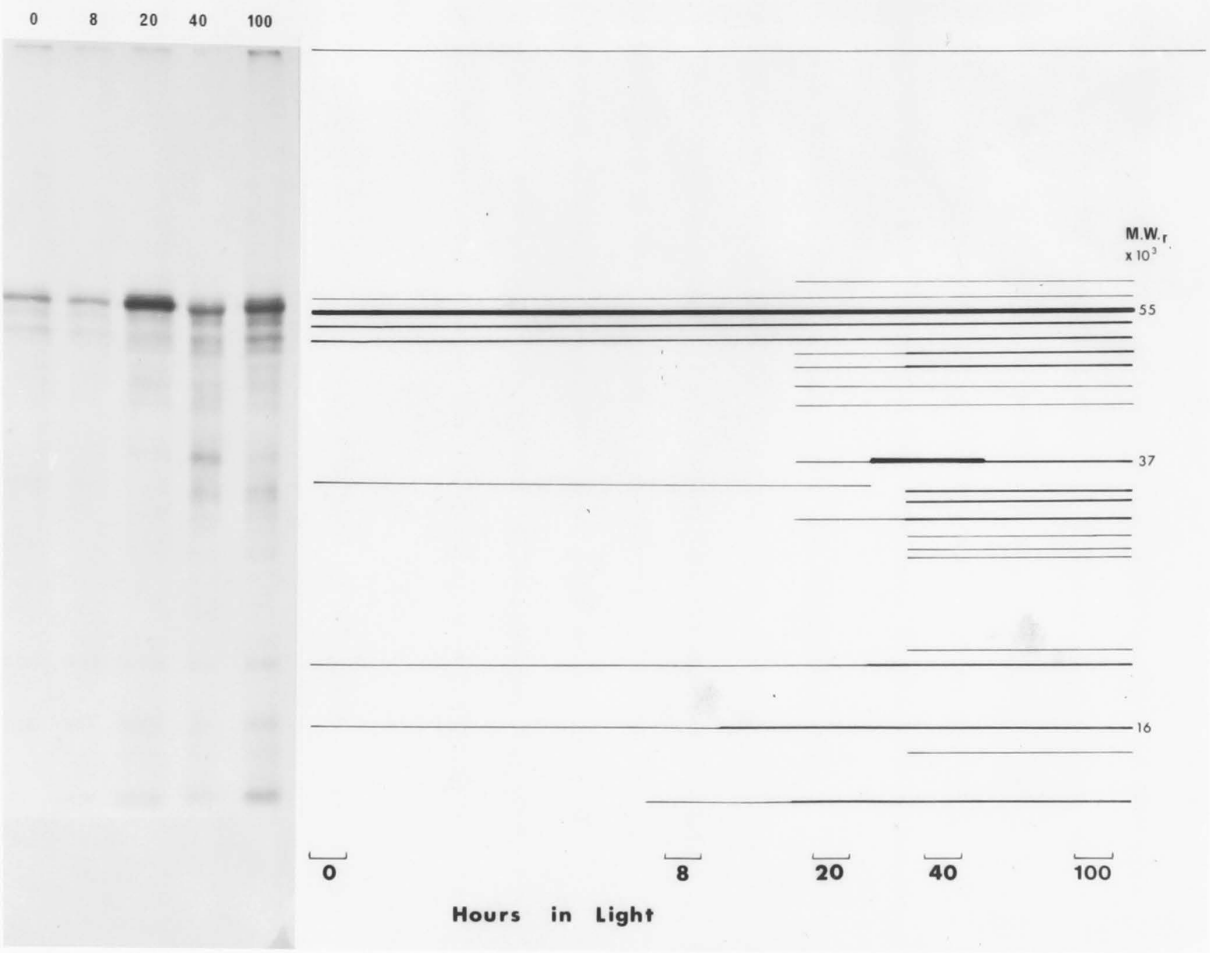
The activity of total plastid polysomes isolated from peas after different times in the light is shown in Figure 3.19. The activity of polysomes fractionated into free and membrane-bound, as described earlier, is also graphed (Figure 3.19). Five µg of polysomes (100 µg/ml) were added to each assay mix.

The low activity of the membrane polysomes is probably a function of the procedure used to prepare these extracts. Grebanier *et al.*, (1978) found a 50 to 70 percent inactivation of chloroplast membrane polysomes when detached from membranes. Despite the reduced activity of the membrane polysomes, the trend shown by each polysome preparation during chloroplast formation is clear. There is a drop in polysome activity in the first eight hours of greening followed by an increase in activity to 45 hours and then a slow decline with aging. The period of greening which yields maximum polysome activity coincides with the period of maximum rate of protein synthesis by the isolated plastids. Since equal amounts of polysomes were used for each assay the fluctuations should reflect changes in the efficiency of translation.

Using total polysomes prepared from pea plastids at different stages of development the fluorograph of synthesized protein in Figure 3.20 was obtained. This fluorograph shows the products of protein synthesis directed by five µg of pea plastid polysomes per assay (100 µg per ml) and fractionated by SDS-polyacrylamide gel electrophoresis. Figure 3.20 demonstrates that the differences, or at least most of the differences, seen in the time series of *in organelle* protein synthesis are reproduced by the plastid polysome run-off assays. The diagrammatic representation of the figure indicates that some polypeptides are synthesised at different times during chloroplast formation.

Figure 3.20. The gel pattern shows the ^{35}S-methionine labelled proteins synthesized by isolated pea plastid polysomes when incubated in the *E. coli* S-30 translation system (5 µg polysomes per 50 µl assay mix). The line drawing emphasises the changes that occur in the polysomal products during the greening of the pea apices (the hours in light are given on a log scale below the drawing).

Figure 3.20 In vitro translation products of pea plastid polysomes. (Fluorograph)



The labelling of polypeptides by the *in vitro* "run-off" of plastid polysomes appears to reflect the changes that occur in protein synthesis by isolated plastids during chloroplast formation. Changes occur in the times and rates of synthesis of various proteins during greening. Such changes can be seen in the accumulation of proteins by the plastid, in the proteins made by isolated plastids and in the proteins made by plastid polysomes *in vitro*. Some of the controlling factors of plastid protein synthesis during greening, must, therefore, operate at or prior to the formation of plastid polysomes.

3.2.3 *The Transcription of Chloroplast DNA during Greening*

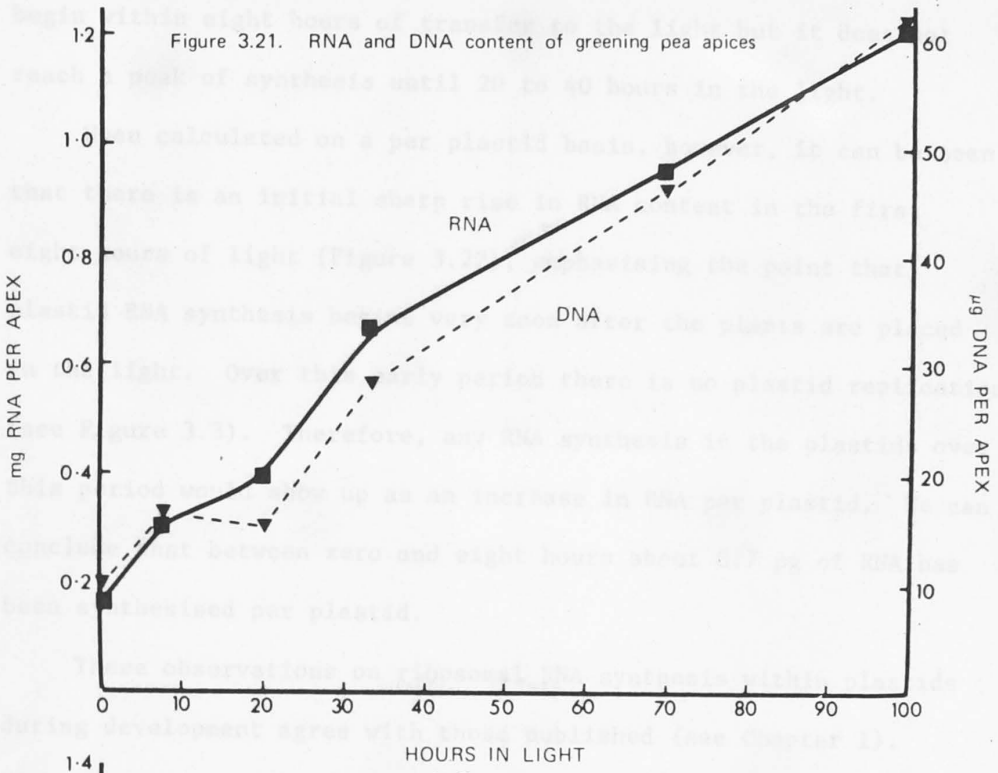
The accumulation of plastid ribosomal RNAs and the template activity of plastid messenger RNAs during chloroplast formation, will be described in this section. A knowledge of these values during greening allows an assessment to be made of the role of transcription of the chloroplast genome in determining the observed changes in protein synthesis as the chloroplasts differentiate (see previous section).

Total apical RNA and DNA both increase at the same rate during greening (Figure 3.21). This increase appears to begin within eight hours of the transfer of seedlings to the light and by 100 hours there has been a six-fold increase in nucleic acid per apex. However, since over the same period there was a 30-fold increase in the apex fresh weight, we can conclude that cell expansion is far more important in raising the apex volume than is cell division as measured by DNA synthesis. Almost exactly the same changes occur in the levels of plastid RNA per apex during development. Again the increase from the etioplast stage to 100 hours after the start of greening is only six-fold (Figure 3.22). The synthesis of plastid RNA appears to

Figure 3.21. Total pea apex nucleic acid content was measured by holding the apices intact during a series of alcohol and ether extractions followed by hydrolysing the nucleic acid out of the tissue with hot perchloric acid. Deoxyribose was determined in the extracts by the diphenylamine reaction and ribose by subtracting the DNA content from total nucleic acid (estimated from the 260 nm absorbance of the extracts).

Figure 3.22. The amount of plastid RNA present in the pea apices was estimated by determining the ratio of plastid to cytoplasmic RNA (by polyacrylamide gel electrophoresis) in RNA extracts from the tissue. Using the data in Figure 3.3 for the number of plastids per apex and knowing the amount of RNA per apex (Figure 3.21), the amount of RNA per plastid was calculated. Each estimation was based on at least ten replicates and the percentage error never exceeded 10 percent.

Figure 3.21. RNA and DNA content of greening pea apices



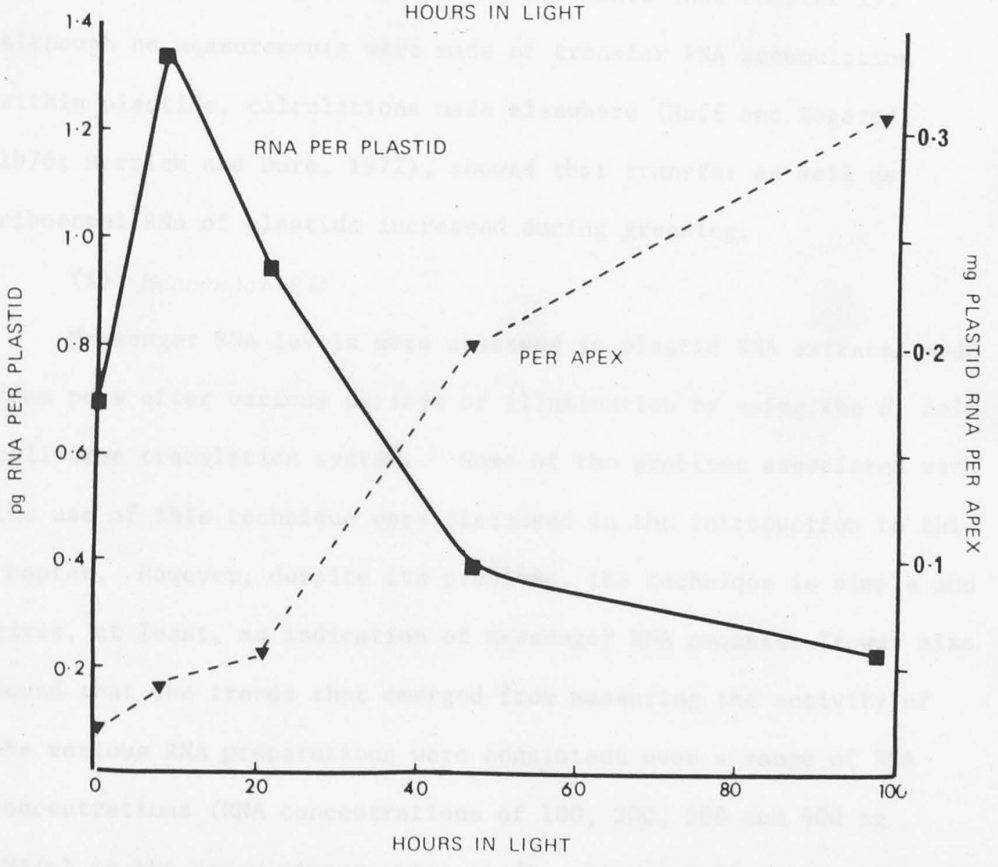


Figure 3.22. Plastid RNA per apex and per plastid

begin within eight hours of transfer to the light but it does not reach a peak of synthesis until 20 to 40 hours in the light.

When calculated on a per plastid basis, however, it can be seen that there is an initial sharp rise in RNA content in the first eight hours of light (Figure 3.22), emphasising the point that plastid RNA synthesis begins very soon after the plants are placed in the light. Over this early period there is no plastid replication (see Figure 3.3). Therefore, any RNA synthesis in the plastids over this period would show up as an increase in RNA per plastid. We can conclude that between zero and eight hours about 0.7 pg of RNA has been synthesised per plastid.

These observations on ribosomal RNA synthesis within plastids during development agree with those published (see Chapter 1). Although no measurements were made of transfer RNA accumulation within plastids, calculations made elsewhere (Haff and Bogorad, 1976; Merrick and Dure, 1972), showed that transfer as well as ribosomal RNA of plastids increased during greening.

(i) *Messenger RNA*

Messenger RNA levels were assessed in plastid RNA extracts made from peas after various periods of illumination by using the *E. coli* cell-free translation system. Some of the problems associated with the use of this technique were discussed in the introduction to this chapter. However, despite its problems, the technique is simple and gives, at least, an indication of messenger RNA amounts. It was also found that the trends that emerged from measuring the activity of the various RNA preparations were consistent over a range of RNA concentrations (RNA concentrations of 100, 200, 300 and 400 µg RNA/ml in the assay mixture were used). Figure 3.23 shows graphs of

Figure 3.23. The *in vitro* template activity of pea plastid RNA preparations was measured in the *E. coli* S-30 translation system and calibrated against a standard chloroplast RNA extract from young spinach leaves. The arbitrary units given for mRNA activity are, therefore, relative to the activity of the spinach chloroplast RNA preparation. These data were corrected to give mRNA activity per plastid and per apex using the data in Figure 3.22 for the RNA content of pea plastids and apices.

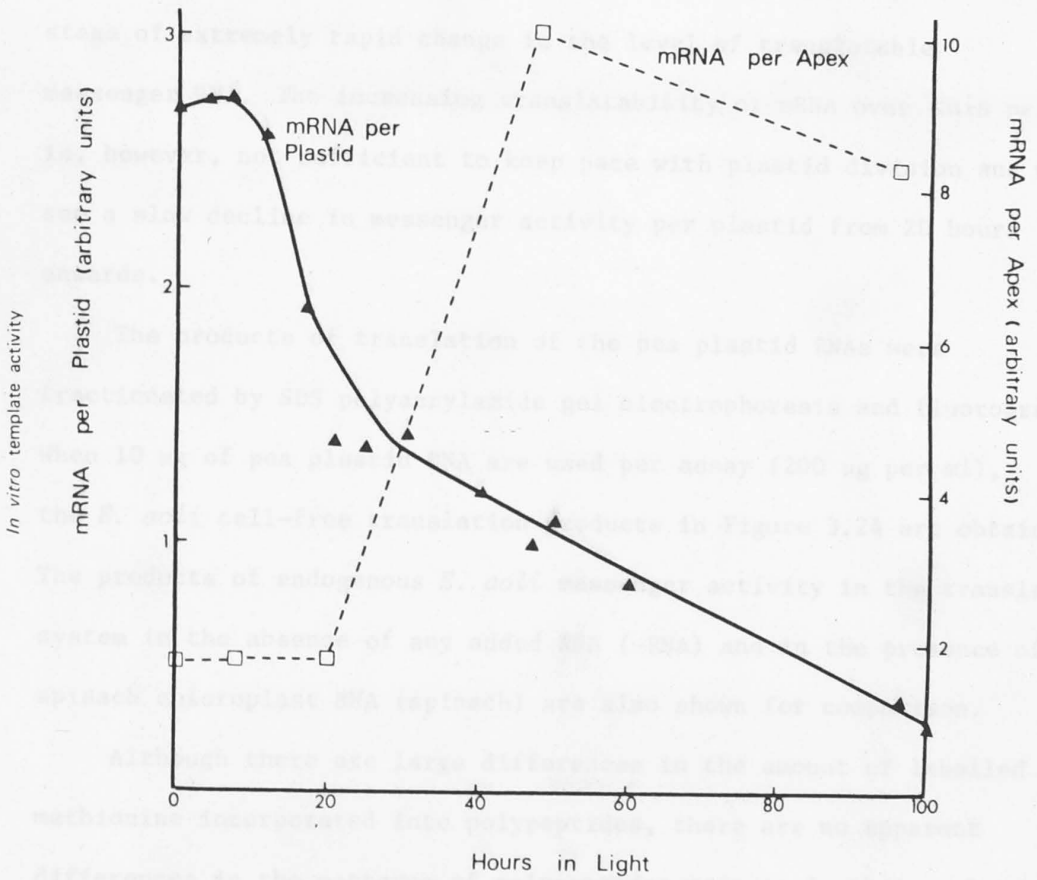


Figure 3.23 Plastid mRNA Activity per Apex and per Plastid (assessed *in vitro*)

the activity of the various plastid RNA preparations with time of development plotted both on a per plastid and a per apex basis. Five µg of plastid RNA were used in each assay (100 µg/ml).

There is no apparent change in the level of messenger RNA per apex during the first 20 hours of chloroplast development. The period from 20 to 40 hours after transfer to the light represents a stage of extremely rapid change in the level of translatable messenger RNA. The increasing translatability of mRNA over this period is, however, not sufficient to keep pace with plastid division and we see a slow decline in messenger activity per plastid from 20 hours onwards.

The products of translation of the pea plastid RNAs were fractionated by SDS polyacrylamide gel electrophoresis and fluorographed. When 10 µg of pea plastid RNA are used per assay (200 µg per ml), the *E. coli* cell-free translation products in Figure 3.24 are obtained. The products of endogenous *E. coli* messenger activity in the translation system in the absence of any added RNA (-RNA) and in the presence of spinach chloroplast RNA (spinach) are also shown for comparison.

Although there are large differences in the amount of labelled methionine incorporated into polypeptides, there are no apparent differences in the patterns of polypeptides made or in their relative amounts. For example, the large decline in LSU synthesis seen in the eight to 20 hour samples is reflected in all the other bands, with the exception of the products of the endogenous messenger RNAs (-RNA). We can conclude, therefore, that there is no obvious preferential transcription of particular messenger RNA species at any time during the course of development. This is in strong contrast to the observations made earlier based on the products of protein synthesis

Figures 3.24 and 3.25. Pea plastid RNA was used as template for the *E. coli* S-30 translation system (10 µg/50 µl assay mix) to give the ^{35}S-methionine labelled proteins shown. Each figure carries channels labelled "-RNA", products synthesized in the absence of added RNA, and "spinach", proteins synthesized by spinach chloroplast RNA. The numbers below each gel channel represent the time (in hours) that the pea apices were allowed to green. The molecular weights of marker proteins appear on the right hand side.

Products of the in vitro translation of pea plastid RNAs.
(Fluorographs)

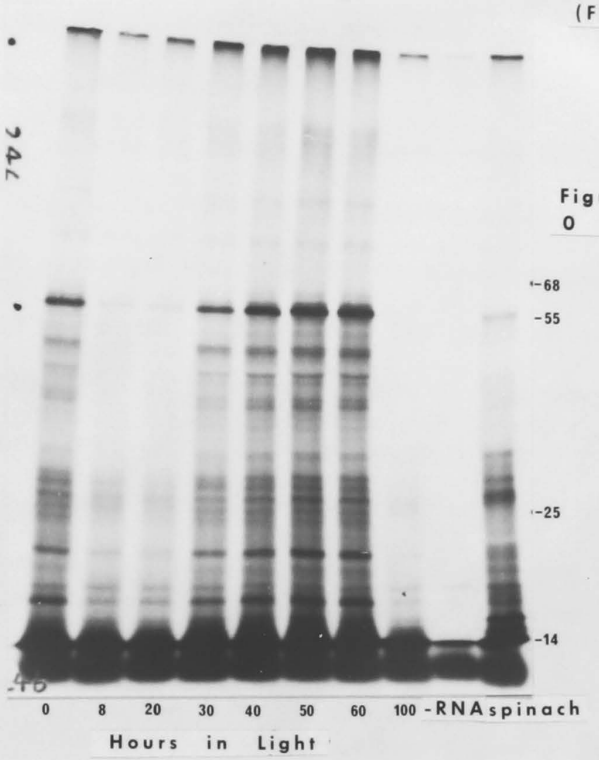


Figure 3.24.
0 to 100 Hours.

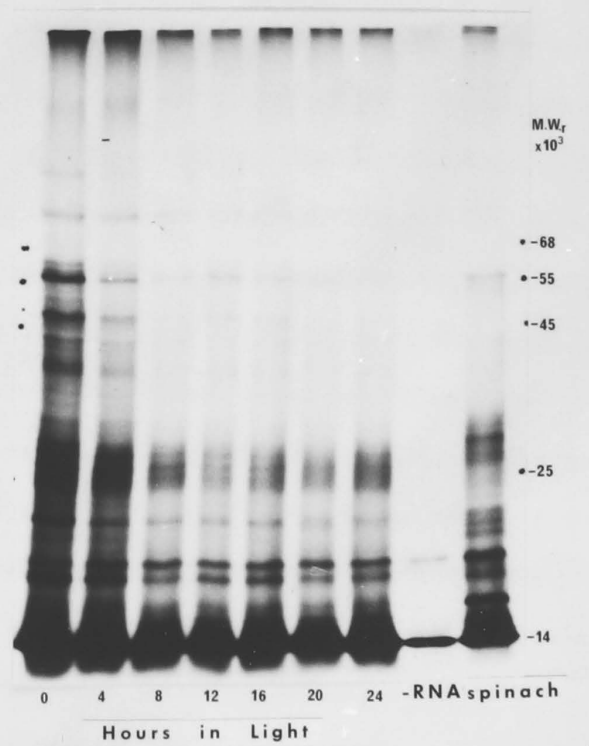


Figure 3.25. 0 to 24 Hours.

by isolated plastids and by polysomes, where changes in the pattern of polypeptides were observed.

Since an equivalent amount of RNA was used for each of the translation assays, the changes in activity reflect a change in the amount of messenger RNA relative to the other plastid RNAs (transfer and ribosomal). Therefore, the large decline in messenger activity in response to light (the 8 and 21 hour samples) reflects a drastic change in the amount of messenger relative to the other forms of RNA. Since there is an increase in the amount of RNA per plastid over the first eight hours of greening (see Figure 3.22 and 3.23) there is actually no decline in mRNA activity per plastid over this period. However, since the major changes appear to occur within the first 20 to 30 hours of illumination, this early period was examined more closely (Figure 3.25). The polypeptides shown in Figure 3.25 were made by the *E. coli* system in response to five µg of added pea plastid RNA. The patterns of products are again the same with increasing times of illumination, the only clear difference between the channels being in intensity of radioactivity. The decline in messenger activity is apparent after only four hours, and reaches minimum activity by eight hours after transfer to the light. Subsequently there is little change in messenger activity until the rise at 30 hours shown in Figure 3.24. Since these estimates of messenger template activity in the *E. coli* translation system are based upon the activity of a constant amount of plastid RNA (ribosomal and transfer, in addition to messenger RNA), the decline in messenger activity represents the preferential accumulation of ribosomal (and perhaps transfer) RNA during greening. Therfore, we can conclude that preferential synthesis of ribosomal RNA begins within four hours after the seedlings have been illuminated. Messenger synthesis does not begin to show a strong response to light until between 24 and thirty hours of illumination.

At no stage, however, is there evidence to suggest that there is a preferential synthesis of certain messenger RNAs.

The other change in the synthesis of messenger RNA occurs late in development between the 60 and 100 hour samples. During this period the leaves are aging and the loss of messenger RNA is presumably due to degradation (Figure 3.23). The messenger activity shown by the 100 hour plastid RNA is largely located in the newly developed leaves at the apex rather than in the older leaves. If the leaves of the pea plants after 100 hours illumination are divided, as shown in Figure 3.26, estimates can be made of the distribution of messenger activity between the mature leaves and the apical region. This comparison is summarised in Table 3.3.

	% Total	
	Apex	Mature Leaves
fresh weight	16	84
RNA (plastid)	18	82
Messenger activity	34	66

Table 3.3. The distribution of messenger activity in old (100 hour) pea plants.

Table 3.3 shows that a disproportionately high level of messenger activity is associated with the apex of the mature pea plants as compared with the old leaves. The products of translation (Figure 3.27) further emphasise the point that the ratio of messenger to ribosomal RNA is several times greater in the apex than the mature leaves.

Figure 3.26. The drawings represent the dissection of the 100 hour greened apex to yield a mature leaf and an apical fraction. These fractions were used to prepare the RNA translated to give the ^{35}S-methionine labelled proteins of Figure 3.27. The amount of RNA added to each 50 µl translation assay is shown and also the position of some molecular weight marker proteins.

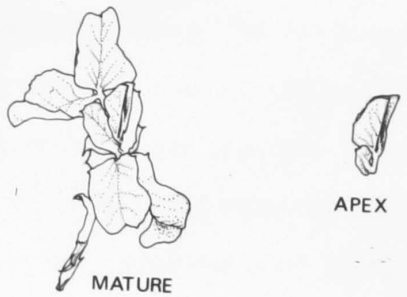


Figure 3.26. The separation of apical from mature leaves of pea seedlings after 100 hours of greening

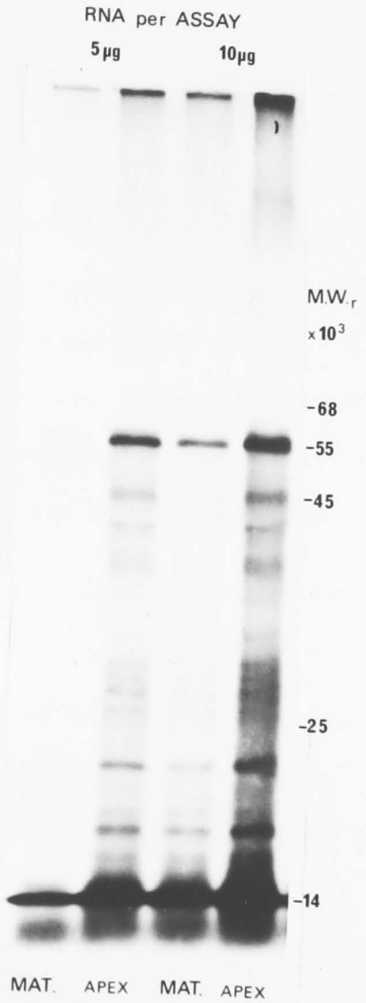


Figure 3.27. Products of the *in vitro* translation of pea chloroplast RNA. Comparison of chloroplast RNA from young and old leaves.

These results imply that the decline in messenger activity shown between 60 and 100 hours of illumination is due to the senescence of the pea leaves and chloroplasts. Newly produced leaves at the apex of these older plants still have high levels of messenger RNA, presumably reflecting active chloroplast protein synthesis.

3.2.4. *The Translation of Pea Plastid Messenger RNAs During Greening*

This section considers the role of translation in the control of plastid protein synthesis, particularly in respect to the synthesis of the LSU protein. It has become apparent, from the results already described, that much of the specificity of protein synthesis shown by the developing pea plastids is not reflected in the *in vitro* translational data obtained using plastid RNA as template. Therefore, one might predict that translational control is involved to some extent in determining the rate and nature of plastid protein synthesis.

(i) *The Number of Ribosomes Associated with Pea Plastid Messenger RNAs.*

One of the fundamental parameters of translation is the size of the polysomes involved in protein synthesis (Palmiter, 1975). It was suggested in the introduction that polysome size was most easily estimated by fractionating polysomes on a sucrose gradient and assaying for the distribution of messenger RNA over the various polysome size classes. The method could be made quantitative either by translating the RNA extracted from the gradient fractions or by allowing the fractionated polysomes to "run-off" in the *E. coli* translation system. Since there are fewer problems with calibration of the "run-off" assays than there are with direct translation assays, the former were used to measure the sizes of pea plastid polysomes.

(a) Polysome size estimation by "run-off" assays.

Figure 3.28 shows the products of run-off assays down a sucrose gradient of polysomes made from pea plastids after 20 hours' illumination. Although there is activity in each of the six fractions taken, some fractions show more activity than others. Also the pattern of polypeptide products varies somewhat depending on the region of the gradient from which the polysomes were recovered. The protein products may be extracted from specific regions of the polyacrylamide gel and the radioactivity in a single band estimated. Since the gradients were isokinetic, the distance moved by the polysomes is directly proportional to their sedimentation velocity (see Appendix I). Consequently, the mean sedimentation coefficient of the polysomes responsible for the synthesis of individual polypeptides can be calculated. By applying the appropriate correction for non-linearity of polysome size with sedimentation coefficient, a mean polysome size can be estimated for a single polypeptide.

The histograms used to calculate the mean sedimentation coefficient of the polysomes synthesising LSU are demonstrated in Figure 3.29. This figure gives the size distribution of pea polysomes synthesising the LSU during the course of pea plastid development. Four other soluble proteins and the proteins made by membrane-bound polysomes (in total) have also been studied in this way. When polysome size is plotted against the time in the light (Figure 3.30), a pattern emerges of changes in polysome size during chloroplast development. Each data point on Figure 3.30 represents the mean of four estimations. Variation never exceeded 10 percent on either side of the mean and was usually less than five percent. We see that the polysomes synthesising soluble proteins show no increase in size until after eight hours of illumination; in fact three of the four plots show

Figure 3.28. Polysomes prepared from 20 hour greened pea apices were separated on an isokinetic sucrose gradient. After centrifugation to pellet the polysomes from each fraction, the *E. coli* S-30 translation system was used to allow the recovered polysomes to "run-off" and synthesize the proteins shown. The products synthesized by the "run-off" of unfractionated polysomes (15 and 10 µg polysomes per assay) and in the absence of added polysomes (-RNA) are given in the right hand channels.

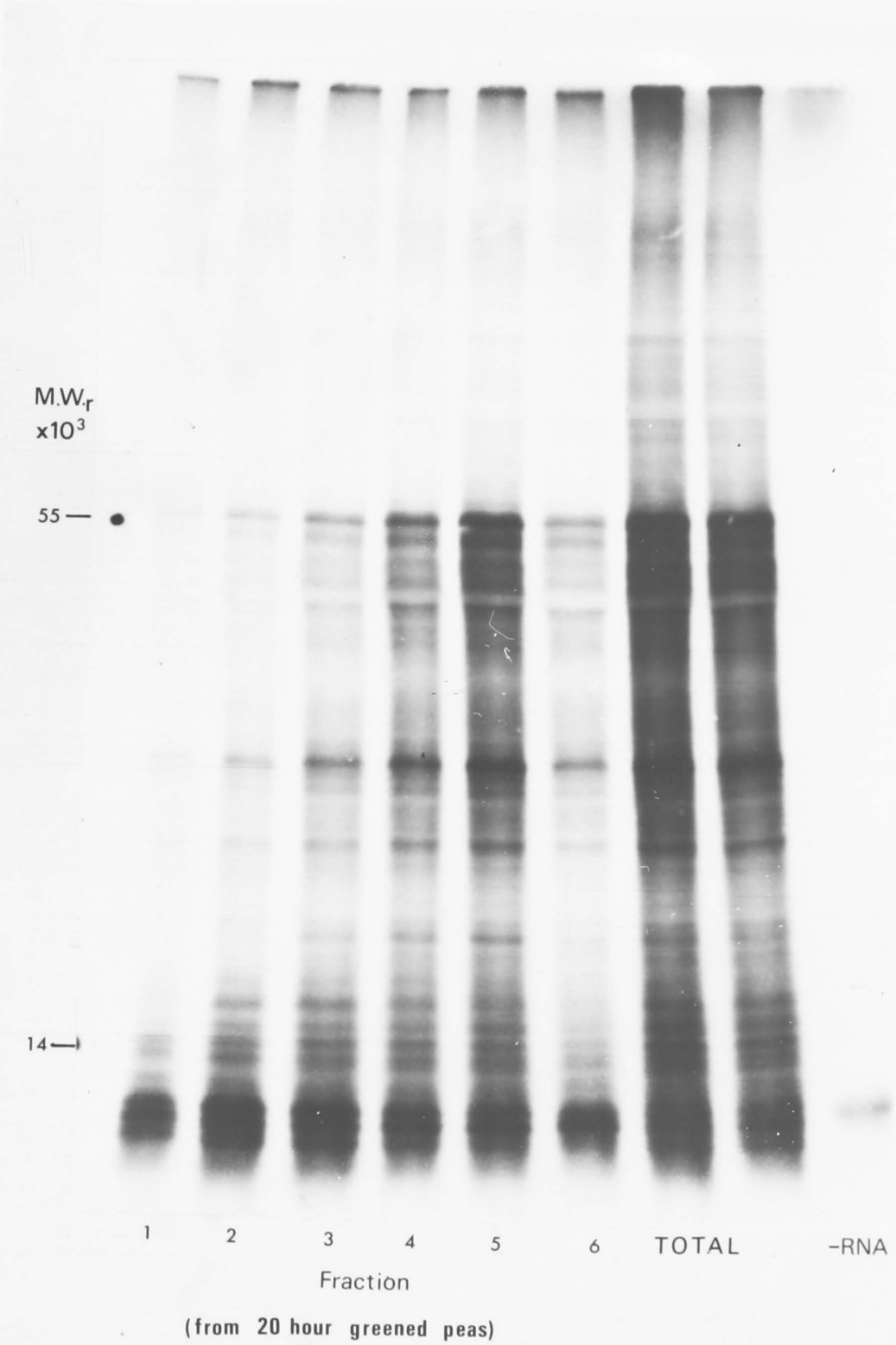


Figure 3.28. Products of the *in vitro* "run-off" of sucrose gradient fractionated pea plastid polysomes.
(Fluorograph of gradient gel)

Figure 3.29. Polysomes separated and recovered as described earlier (see Figure 3.28) were assayed for the size distribution of LSU-synthesizing polysomes. The proportion of LSU synthesis by each fraction relative to total LSU synthesis is plotted on the left hand axis. The developmental stage of the plastids used to provide the polysomes for each histogram and the locations of the plastid mono-, di-, tri- and tetra- polysomes are indicated.

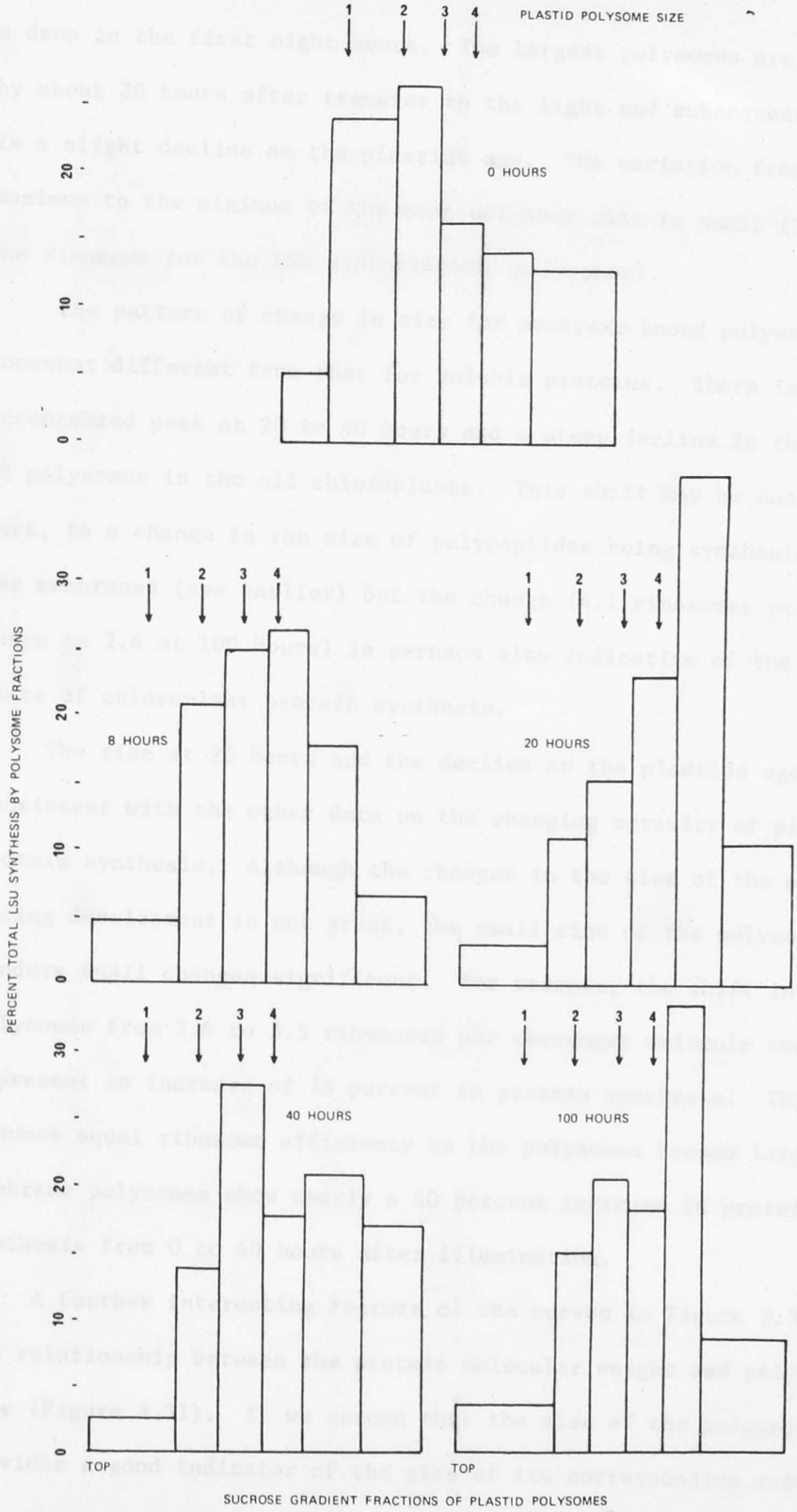


Figure 3.29. The distribution of LSU-synthesising polysomes on sucrose gradient fractionated pea plastid polysomes.

a drop in the first eight hours. The largest polysomes are formed by about 20 hours after transfer to the light and subsequently there is a slight decline as the plastids age. The variation from the maximum to the minimum of the mean polysome size is small (less than one ribosome for the LSU synthesising polysomes).

The pattern of change in size for membrane-bound polysomes is somewhat different from that for soluble proteins. There is a more accentuated peak at 20 to 40 hours and a steep decline in the size of polysomes in the old chloroplasts. This shift may be due, in part, to a change in the size of polypeptides being synthesised on the membranes (see earlier) but the change (4.1 ribosomes per mRNA at 40 hours to 2.4 at 100 hours) is perhaps also indicative of the changing state of chloroplast protein synthesis.

The rise at 20 hours and the decline as the plastids age is consistent with the other data on the changing activity of plastid protein synthesis. Although the changes in the size of the polysomes during development is not great, the small size of the polysome renders small changes significant. For example, the shift in LSU polysomes from 2.6 to 3.5 ribosomes per messenger molecule could represent an increase of 35 percent in protein synthesis. This assumes equal ribosome efficiency as the polysomes become larger. Membrane polysomes show nearly a 60 percent increase in protein synthesis from 0 to 40 hours after illumination.

A further interesting feature of the curves in Figure 3.30 is the relationship between the protein molecular weight and polysome size (Figure 3.31). If we assume that the size of the polypeptide provides a good indicator of the size of its corresponding messenger RNA, then certain predictions can be made based upon Figure 3.31. The size of the polysomes could be either dependent or independent

Figures 3.30 and 3.31. Polysomes fractionated and analysed as shown in Figures 3.28 and 3.29 were used to assess the mean size of polysomes synthesizing a range of proteins. Four proteins made by free polysomes (55, 40, 19 and 12 thousand dalton polypeptides) and the mean size of the active membrane-associated polysome are plotted.

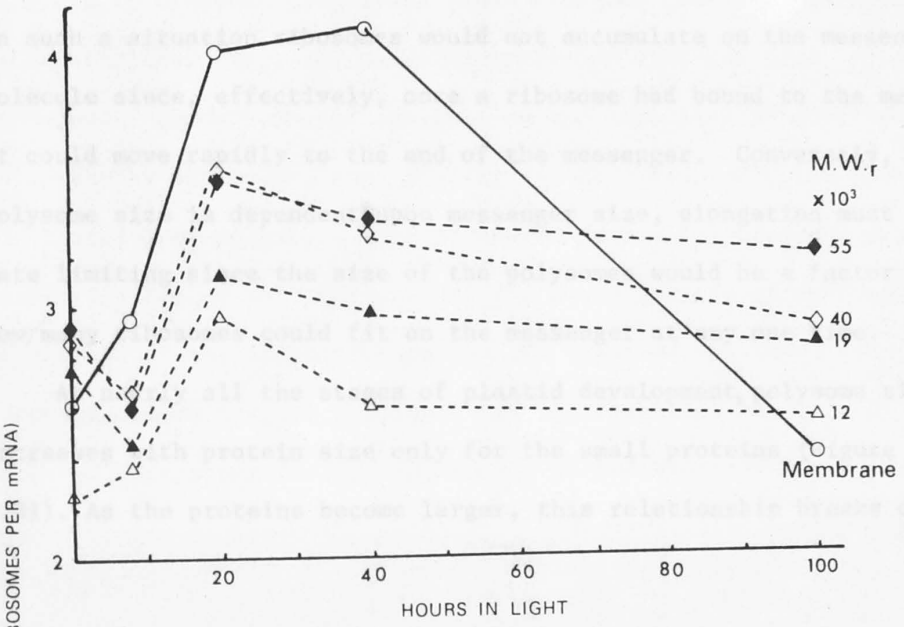


Figure 3.30. The size of pea plastid polysomes during greening

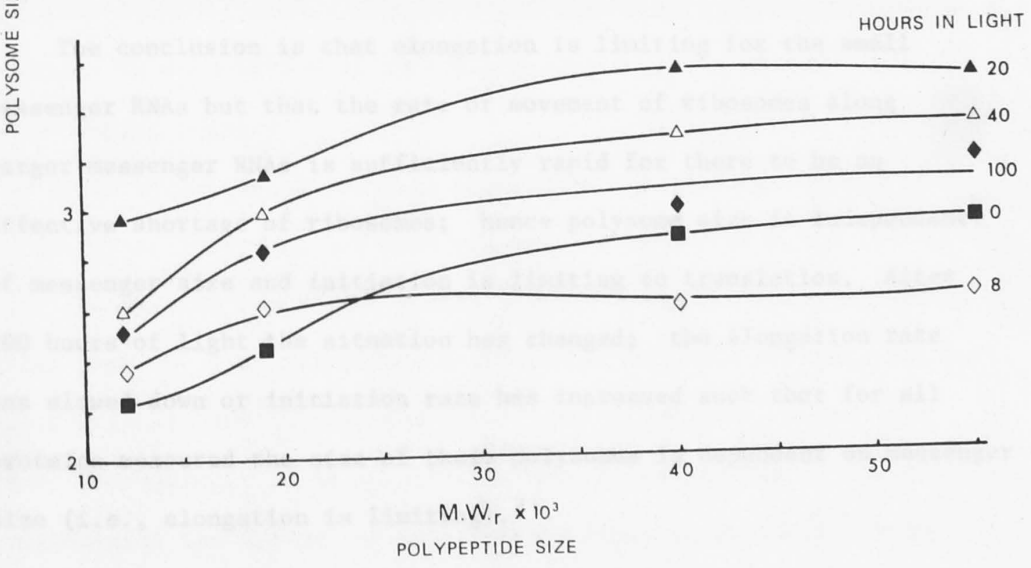


Figure 3.31. Relationship between polysome size and polypeptide size

of the size of their messenger RNAs. If the polysome size is independent of messenger size then initiation must be rate limiting for translation. In such a situation ribosomes would not accumulate on the messenger molecule since, effectively, once a ribosome had bound to the message it could move rapidly to the end of the messenger. Conversely, when polysome size is dependent upon messenger size, elongation must be rate limiting since the size of the polysomes would be a factor of how many ribosomes could fit on the messenger at any one time.

At nearly all the stages of plastid development, polysome size increases with protein size only for the small proteins (Figure 3.31). As the proteins become larger, this relationship breaks down.

The conclusion is that elongation is limiting for the small messenger RNAs but that the rate of movement of ribosomes along larger messenger RNAs is sufficiently rapid for there to be an effective shortage of ribosomes; hence polysome size is independent of messenger size and initiation is limiting to translation. After 100 hours of light the situation has changed; the elongation rate has slowed down or initiation rate has increased such that for all proteins measured the size of their polysomes is dependent on messenger size (i.e., elongation is limiting).

(b) Ferritin-antibody labelling of polysomes synthesizing the large subunit of RuBPCase.

The principle problem with the data just presented is determining their reliability. It is difficult, firstly, to establish the validity of using polysome preparations that may have suffered degradation during preparation and secondly, to assess the reliability

of the assays used for messenger activity down the polysome profile. In order to overcome these, and other problems described earlier, an alternative technique for measuring polysome size was used. The technique depends upon the recognition and labelling of specific polysomes by antibodies made against, in this case, the LSU protein.

Total leaf polysomes were incubated on ice for one hour with ferritin-labelled antibody against the large subunit of RuBPCase At the end of the incubation period the labelled polysomes were fractionated on sucrose gradients and the fractions examined by electron microscopy for the appearance of the electron-dense protein, ferritin, associated with ribosomes. Where such associations were seen, the presence of a nascent polypeptide of the large subunit protein was presumed. Figure 3.32 shows a series of micrographs prepared for pea and wheat total leaf polysomes labelled with ferritin antibody (a description of each micrograph is given on the accompanying page).

This technique has several advantages; it is independent of messenger activity and one does not need to depend upon the reliability of a translation system for quantitation; it is extremely sensitive (less than 10 μg of polysomes is sufficient to yield 20 fractions for observation); and it provides other data in addition to polysome size information. It is possible to measure the number of polysomes synthesising the protein of interest and hence to derive an estimate of the number of messengers present in the polysome mixture. Also the technique has the ability to examine a single, and possibly minor, component of a mixture of polysomes. For example, in the examination of large subunit-synthesising polysomes, it is feasible

Figure 3.32. Electron Micrographs of Total Pea and Wheat Polysomes Labelled with Ferritin-Anti LSU.

b = ferritin-anti-LSU bound to the ribosomes

u = ferritin-anti-LSU that has not bound to the ribosomes

A to D

A. Polysomes prepared from 10 day old 2nd wheat leaf, labelled with ferritin-anti-bean-LSU. This polysome fraction was taken from a sucrose gradient in the region 6 to 10-mer polysome size. A number of large polysomes can be seen; most have not been labelled by the ferritin-antibody.

B. An enlargement of part of A showing the labelling of a disome with five ferritin particles. The number of ferritin molecules seen associated with the ribosomes may correspond to the length of the nascent LSU chain; long enough to have three antigenic sites in one case and two sites in the second case. It is also possible that the structure shown was produced by the binding of a ferritin-antibody aggregate to a single nascent protein antigenic site.

C and D. Polysomes prepared from 20 hour greened pea apices and labelled with ferritin-anti-spinach LSU. As for A and B above the polysomes were taken from the 6 to 10-mer region of a polysome profile.

D shows an enlargement of part of C with a disome labelled by three ferritin molecules. A large polysomal structure also appears to have been labelled.

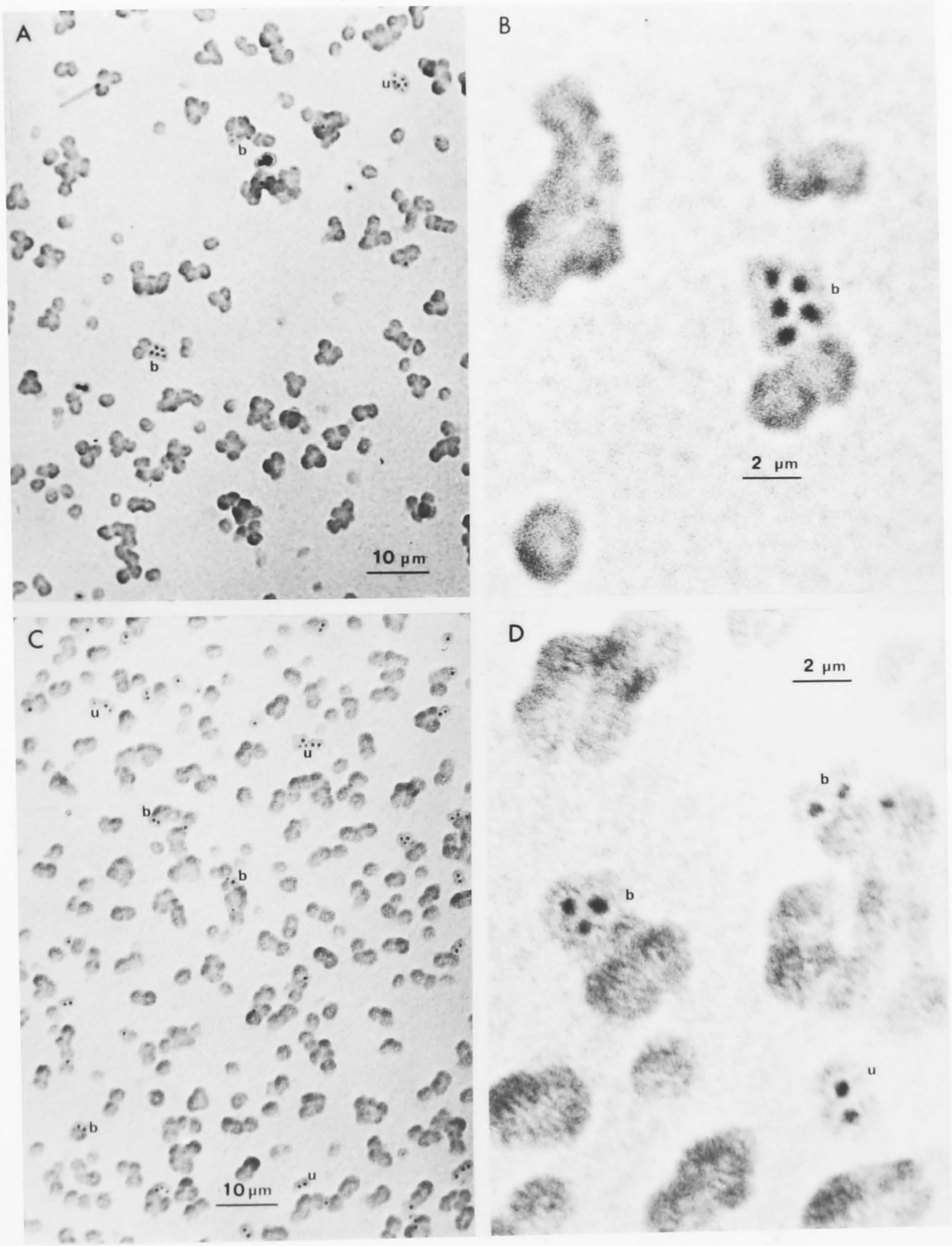
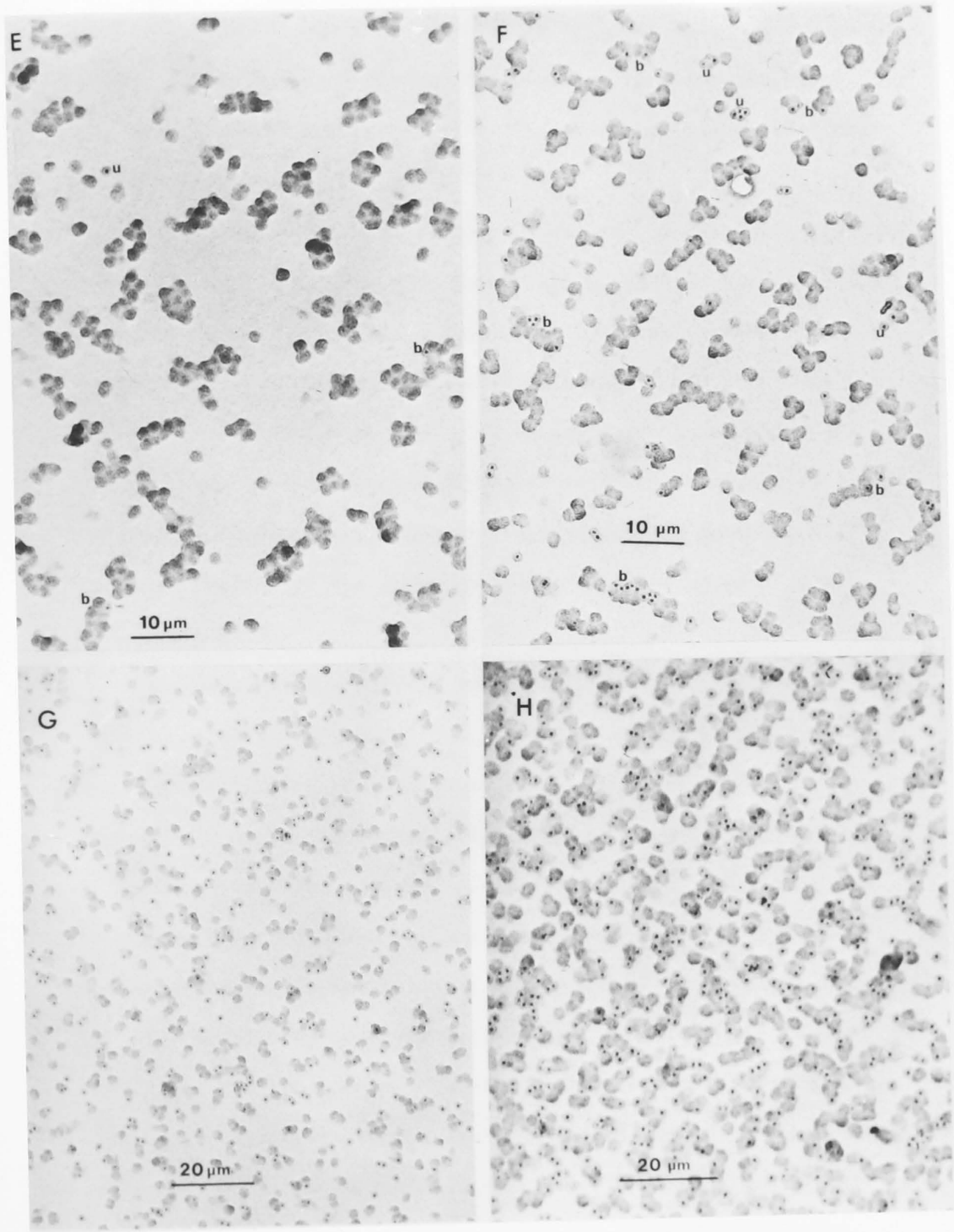

A
u
b
b
10 µm
B
b
2 µm
C
u
u
b
b
b
u
10 µm
D
2 µm
b
b
u

Figure 3.32. E to H.

E and G are micrographs of etiolated pea apex polysomes labelled with ferritin-anti-spinach LSU. Few of the large polysomes in E have been labelled whereas the small polysomes in G (one and two-mers mainly) have been extensively labelled.

H and F show the labelling of large and small (respectively) polysomes from the 2nd wheat leaf 9 days after sowing. Again not many large polysomes are labelled by the antibody but LSU synthesising polysomes appear to be more common amongst the large wheat polysomes (F) than amongst those of the etiolated peas (E). The small wheat polysomes (H), as with their pea counterparts (G), have been extensively labelled but many free, unbound ferritin particles are still visible. The preparation shown in (H) emphasises the problems that arise from the random coincidence of the ferritin with robosomes if the concentration of the two types of particles is too great. For scoring the proportion of ribosomes labelled by the ferritin-antibody it would be necessary to dilute the polysome preparation shown in H, at least five-fold.


E
u
b
b
10 µm
F
b
u
u
b
b
u
b
10 µm
b
G
20 µm
H
20 µm

to use a preparation of total leaf polysomes. These polysome preparations can be made far more rapidly than can those from isolated plastids and this helps avoid degradation and provides more RNA to act as a shield against degradation, thus avoiding many of the problems with the preparation of plastid polysomes described earlier (section 3.2.1. (iii)).

The technique also has its problems. It is more difficult and time consuming than messenger quantitation, and one cannot be sure that all nascent polypeptide chains are being recognised and bound by the antibody. For example, the chain may be too short and not carry the necessary antigenic site or antigenic recognition may depend upon the conformation of the protein. A further problem is avoiding non-specific binding of the antibody and coincidental juxtapositioning of the ferritin-antibody with ribosomes on the microscope grid.

In the case of the large subunit of RuBPCase, this last problem is easily corrected for. Total polysomes from the roots of wheat plants were used as a control for non-specific binding. Figure 3.33 shows the distribution of ferritin-antibody labelled polysomes down wheat leaf and root profiles. By subtracting the root from the leaf labelling one can arrive at a more reliable estimate for the distribution of large subunit-synthesising polysomes.

The problem of deciding whether full labelling has occurred is more difficult to assess. However, if the extent of labelling is calculated, it appears that between 70 and 80 percent of the plastid polysomes are labelled by anti-large subunit ferritin-antibody. These values are based on estimates of the percentage of total ribosomes labelled and a knowledge of the distribution of chloroplast ribosomes down the gradients (assessed by estimating 16S and 18S RNA,

Figure 3.33. Polysomes prepared from the second leaves of 10 day old wheat seedlings were labelled with ferritin-anti-bean LSU. The gradient profile ($O.D._{260}$) and the percent of ribosomes labelled with ferritin for both a wheat leaf and root polysome preparation are shown. The labelling of polysomes with the ferritin antibody was scored through the electron microscope using sucrose gradient fractions of the labelled polysomes.

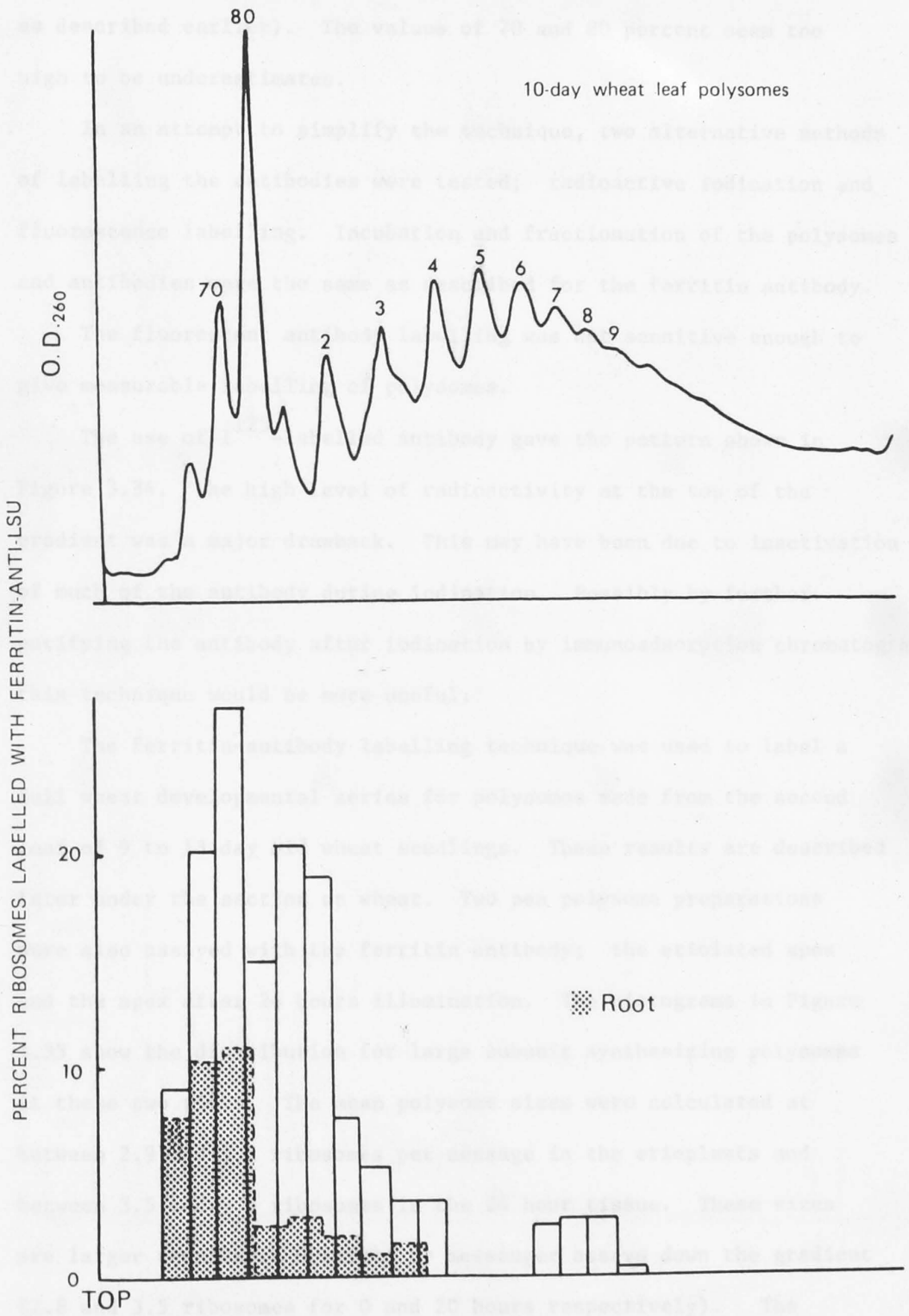


Figure 3.33. Ferritin-anti-LSU labelling of wheat leaf and root polysomes

as described earlier). The values of 70 and 80 percent seem too high to be underestimates.

In an attempt to simplify the technique, two alternative methods of labelling the antibodies were tested; radioactive iodination and fluorescence labelling. Incubation and fractionation of the polysomes and antibodies were the same as described for the ferritin antibody.

The fluorescent antibody labelling was not sensitive enough to give measurable labelling of polysomes.

The use of I^{125}-labelled antibody gave the pattern shown in Figure 3.34. The high level of radioactivity at the top of the gradient was a major drawback. This may have been due to inactivation of much of the antibody during iodination. Possibly by further purifying the antibody after iodination by immunoadsorption chromatography, this technique would be more useful.

The ferritin-antibody labelling technique was used to label a full wheat developmental series for polysomes made from the second leaf of 9 to 13 day old wheat seedlings. These results are described later under the section on wheat. Two pea polysome preparations were also assayed with the ferritin antibody; the etiolated apex and the apex after 24 hours illumination. The histograms in Figure 3.35 show the distribution for large subunit synthesizing polysomes at these two times. The mean polysome sizes were calculated at between 2.9 and 3.2 ribosomes per message in the etioplasts and between 3.5 and 3.9 ribosomes in the 24 hour tissue. These sizes are larger than those obtained by messenger assays down the gradient (2.8 and 3.5 ribosomes for 0 and 20 hours respectively). The greater size could be due to the degradation of some of the plastid polysomes used for the messenger assay technique or to a distortion

Figure 3.34. Wheat leaf polysomes labelled with ^{125}I-anti LSU were separated on a sucrose gradient to give the profile and distribution of radioactivity shown.

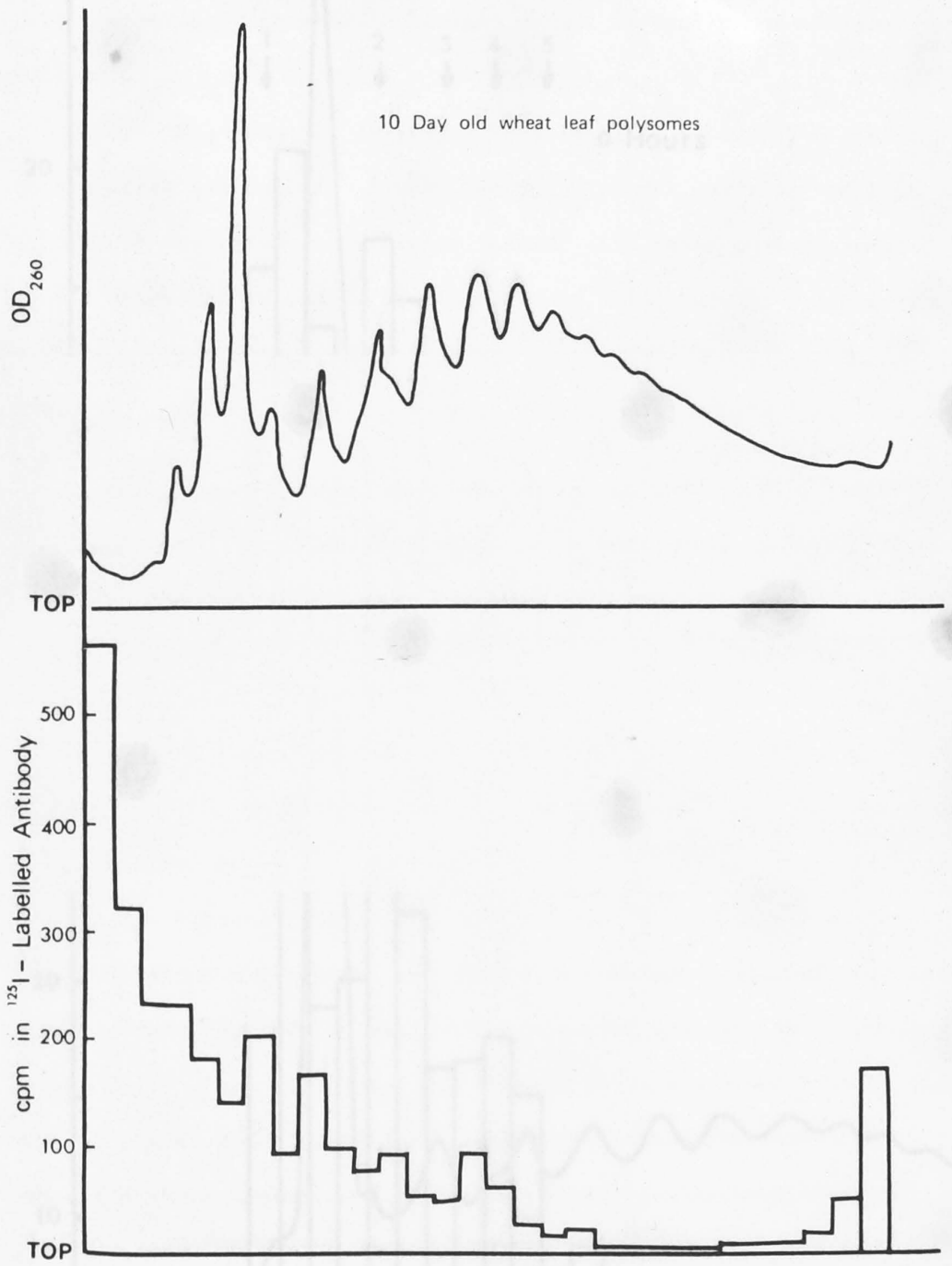


Figure 3.34. ^{125}I-anti LSU labelling of wheat polysomes

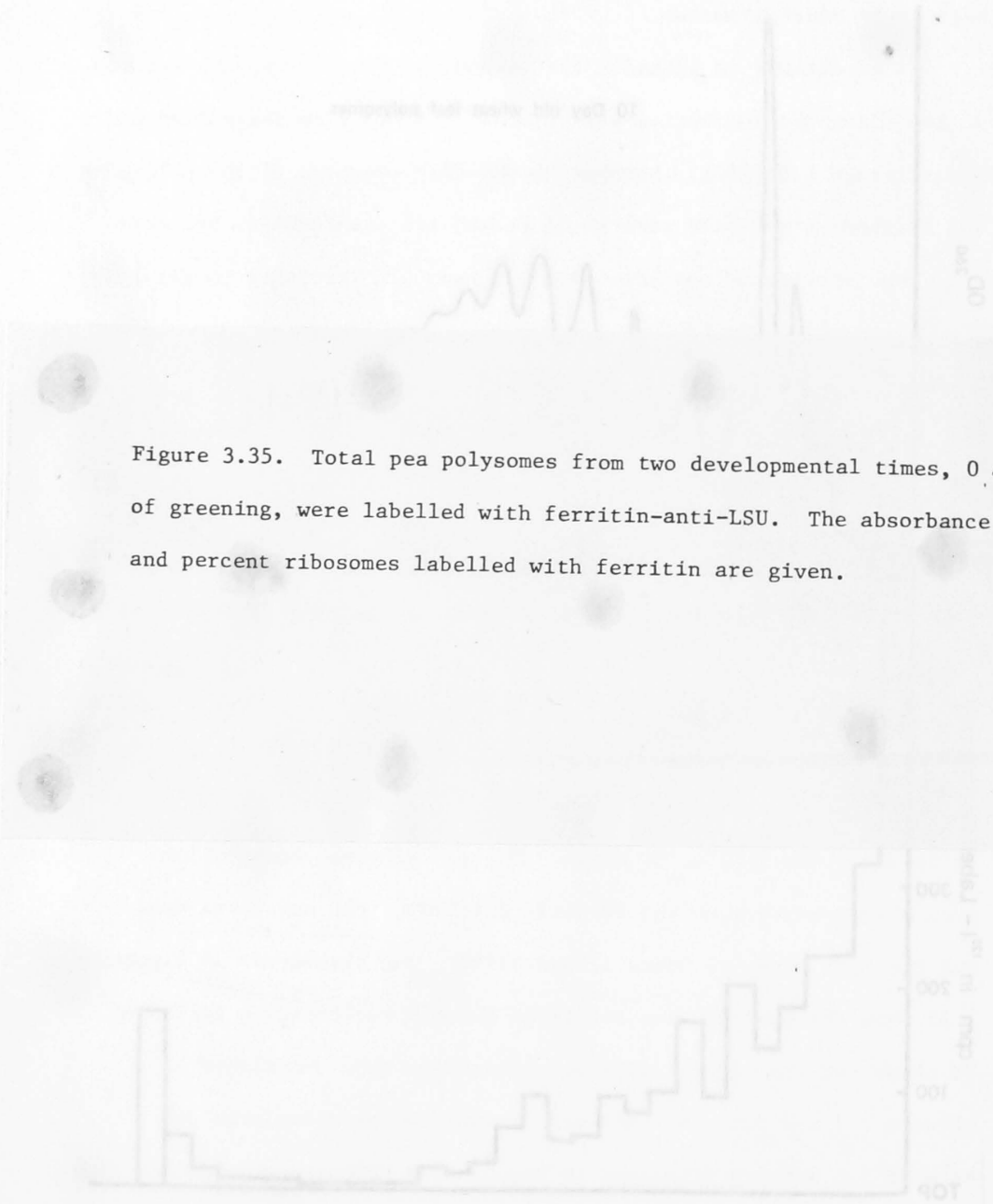


Figure 3.35. Total pea polysomes from two developmental times, 0 and 24 hours of greening, were labelled with ferritin-anti-LSU. The absorbance profile and percent ribosomes labelled with ferritin are given.

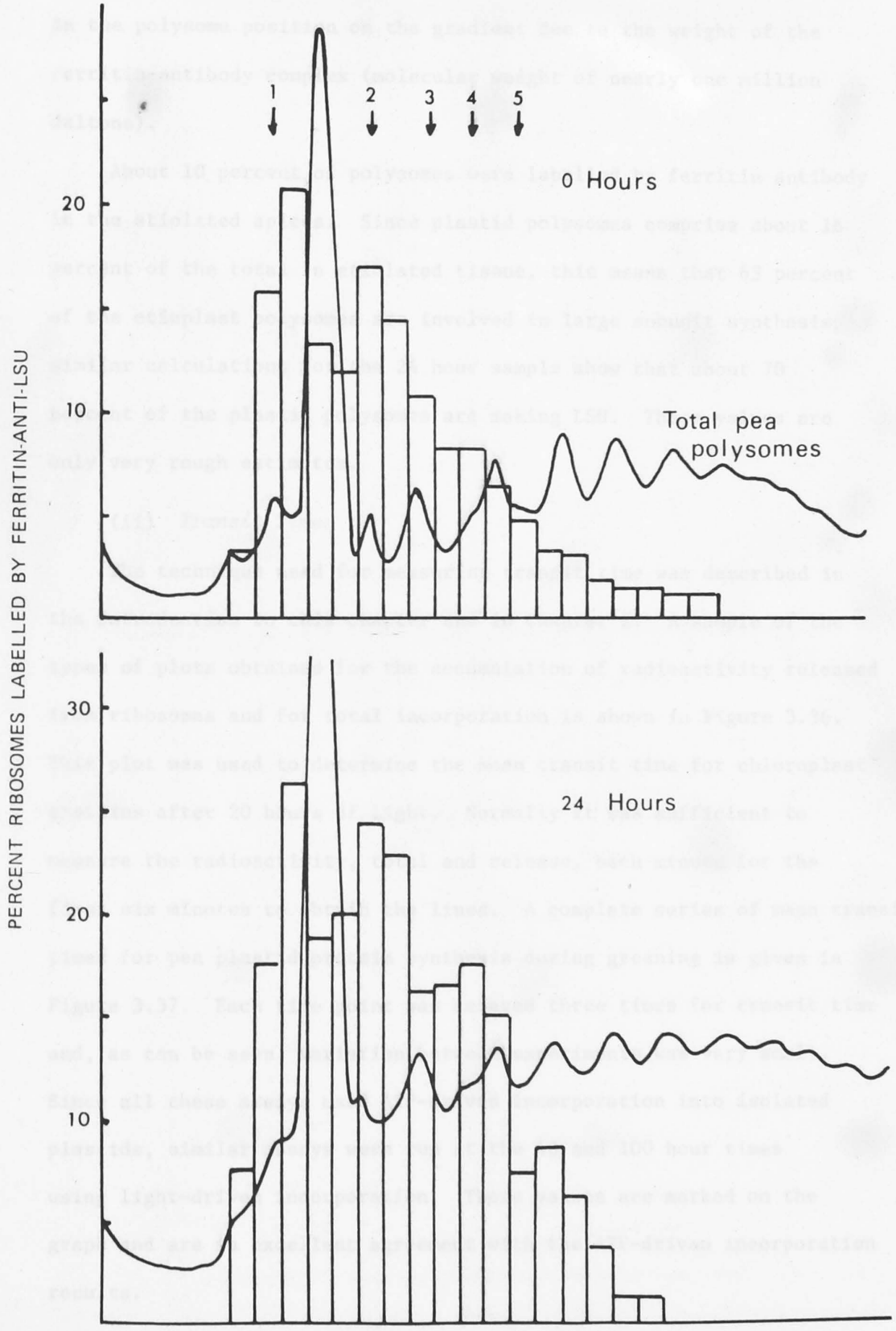


Figure 3.35. Ferritin-anti-LSU labelling of pea polysomes

in the polysome position on the gradient due to the weight of the ferritin-antibody complex (molecular weight of nearly one million daltons).

About 10 percent of polysomes were labelled by ferritin antibody in the etiolated apices. Since plastid polysomes comprise about 16 percent of the total in etiolated tissue, this means that 63 percent of the etioplast polysomes are involved in large subunit synthesis; similar calculations for the 24 hour sample show that about 70 percent of the plastid polysomes are making LSU. These values are only very rough estimates.

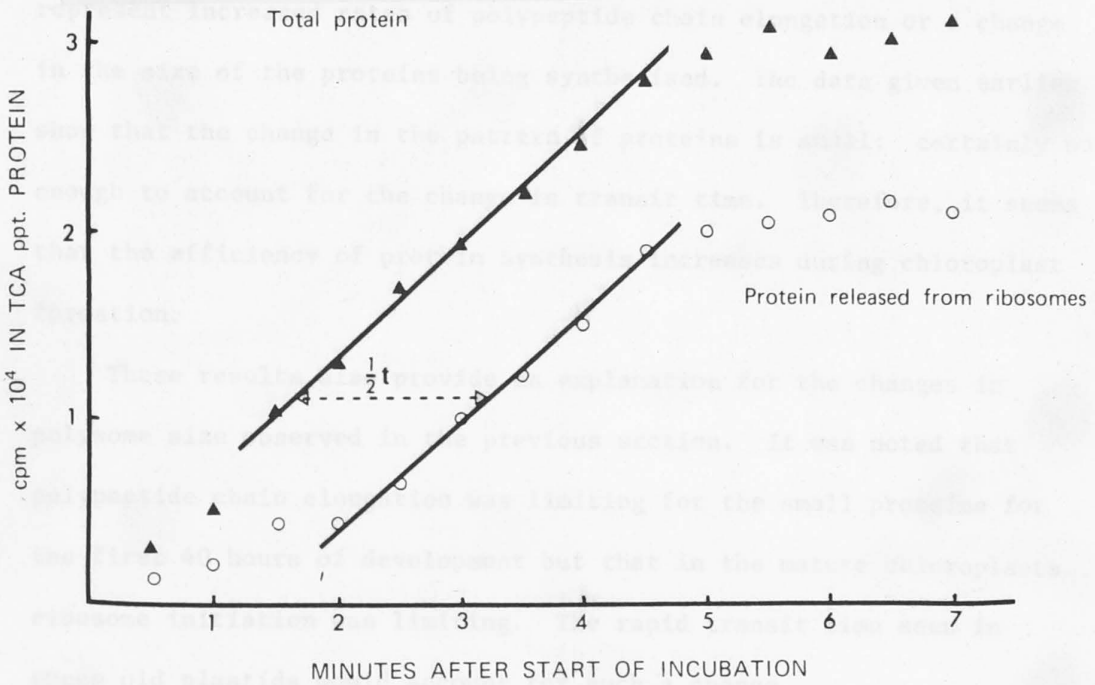
(ii) *Transit Time:*

The technique used for measuring transit time was described in the introduction to this chapter and in Chapter 2. A sample of the types of plots obtained for the accumulation of radioactivity released from ribosomes and for total incorporation is shown in Figure 3.36. This plot was used to determine the mean transit time for chloroplast proteins after 20 hours of light. Normally it was sufficient to measure the radioactivity, total and release, each minute for the first six minutes to obtain the lines. A complete series of mean transit times for pea plastid protein synthesis during greening is given in Figure 3.37. Each time point was assayed three times for transit time and, as can be seen, variation between experiments was very small. Since all these assays used ATP-driven incorporation into isolated plastids, similar assays were run at the 40 and 100 hour times using light-driven incorporation. These values are marked on the graph and are in excellent agreement with the ATP-driven incorporation results.

Figure 3.36. Pea plastids were incubated with ^{35}S-methionine and ATP (as the energy source) for various periods. At 30 second intervals half the plastids were lysed in Triton X-100 and the polysomes pelleted, the supernatant fraction gave a measure of the label in released protein (not associated with the polysomes). The remaining half of the preparation was used to measure the label incorporated into total TCA precipitable protein. The difference between the two lines is equal to one half the mean plastid ribosome transit time (see Figure 3.1).

Figure 3.37. Using plots of the type illustrated in Figure 3.36 the mean ribosome transit time was estimated during pea plastid development. For the mature chloroplasts (40 and 100 hours) two energy sources were used to drive protein synthesis (closed circles - ATP, open circles - light driven).

Figure 3.36. Estimation of mean chloroplast ribosome transit time for 20 hour greened pea apices.



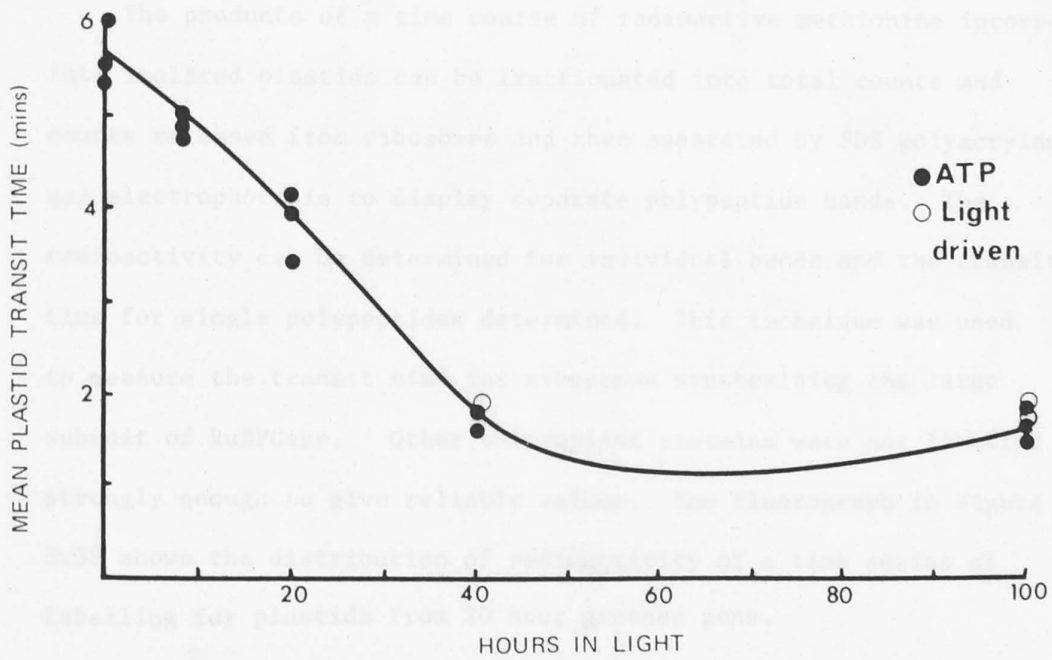


Figure 3.37. Mean pea plastid transit times during greening

The transit time for plastid ribosomes decreases nearly four-fold during chloroplast development. This change could either represent increased rates of polypeptide chain elongation or a change in the size of the proteins being synthesised. The data given earlier show that the change in the pattern of proteins is small; certainly not enough to account for the change in transit time. Therefore, it seems that the efficiency of protein synthesis increases during chloroplast formation.

These results also provide an explanation for the changes in polysome size observed in the previous section. It was noted that polypeptide chain elongation was limiting for the small proteins for the first 40 hours of development but that in the mature chloroplasts ribosome initiation was limiting. The rapid transit time seen in these old plastids would account for such a change.

The products of a time course of radioactive methionine incorporation into isolated plastids can be fractionated into total counts and counts released from ribosomes and then separated by SDS polyacrylamide gel electrophoresis to display separate polypeptide bands. The radioactivity can be determined for individual bands and the transit time for single polypeptides determined. This technique was used to measure the transit time for ribosomes synthesising the large subunit of RuBPCase. Other chloroplast proteins were not labelled strongly enough to give reliable values. The fluorograph in Figure 3.38 shows the distribution of radioactivity of a time series of labelling for plastids from 20 hour greened peas.

The mean transit time for ribosomes synthesising the large subunit for the pea plastid developmental series is shown in Table 3.4.

Figure 3.38. Pea plastid proteins were fractionated into total protein and protein released from polysomes over a time course of *in organelle* labelling. The protein preparations were separated by SDS-polyacrylamide-gradient gel electrophoresis and fluorographed to give the patterns shown. The gel channels are labelled for the incubation time (in minutes) and for the type of preparation, "total" plastid protein or protein "released" from polysomes.

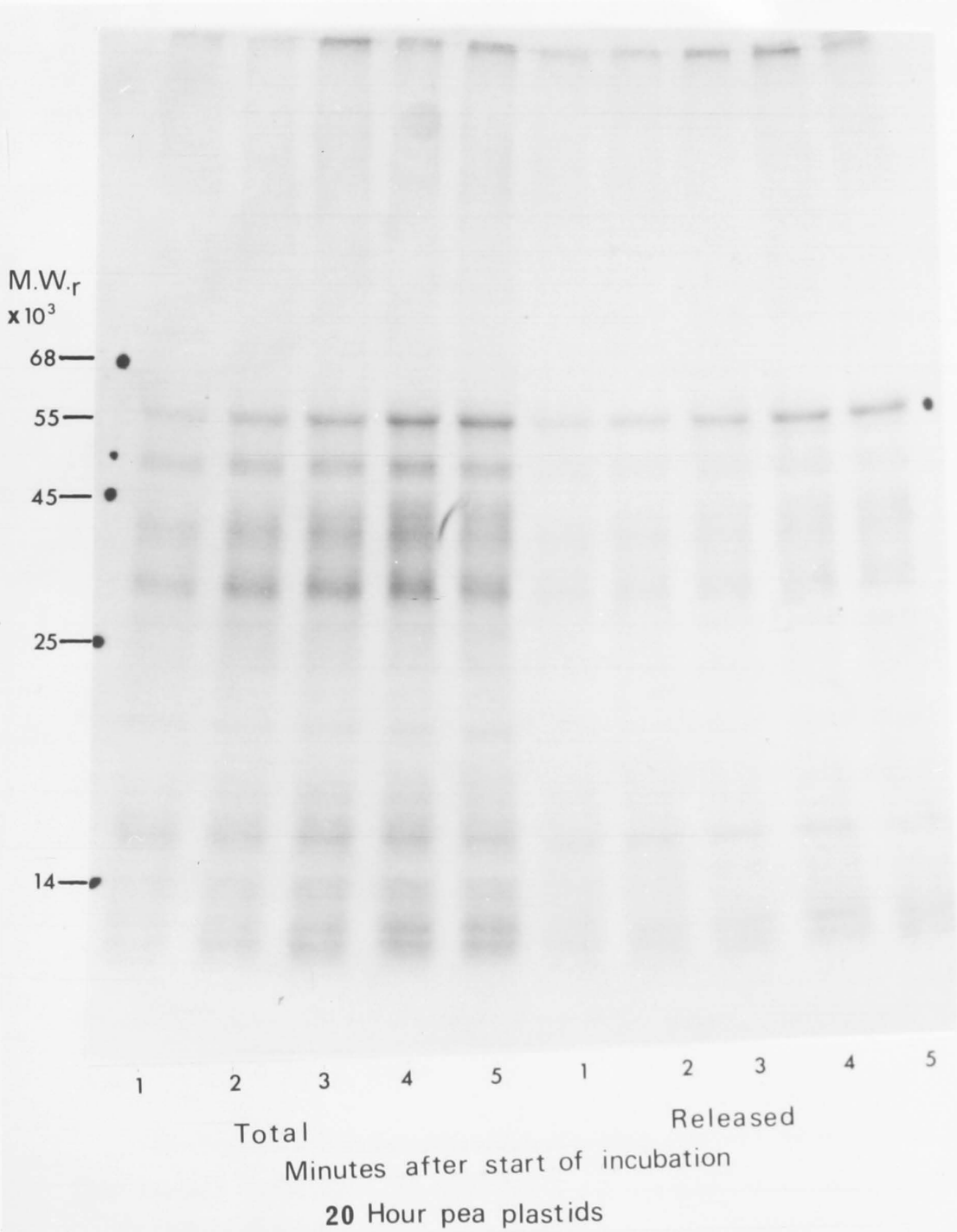


Figure 3.38. Labelling of pea plastid polysomes for transit time estimations (Fluorograph of gradient gel)

Hours in light	Mean chloroplast ribosome transit time (mins)	LSU-mean transit time (mins)	Rate of LSU chain elongation (amino acids/sec)
0	5.7 ± 0.3	1.7 ± 0.3	4.9
8	4.8 ± 0.2	2.7 ± 0.2	3.1
20	4.1 ± 0.1	1.7 ± 0.2	4.9
40	1.6 ± 0.1	2.2 ± 0.2	3.8
80	1.7 ± 0.1	2.3 ± 0.3	3.6

Table 3.4. Transit times and rate of polypeptide chain elongation of the large subunit of RuBPCase during pea chloroplast development.

The values given in Table 3.4 for mean chloroplast ribosome transit time are those used in Figure 3.37. Each "large subunit" transit time measurement is based on five experiments; the standard errors are indicated. Since the size of the LSU polypeptide is known, the rate of polypeptide chain elongation can be calculated.

$$\text{rate of polypeptide chain elongation (amino acids per second)} = \frac{\text{transit time (in seconds)}}{\text{number of amino-acids per polypeptide.}}$$

(these are about 500 amino acids in the large subunit polypeptide, Kawashima and Wildman, 1970).

The data in Table 3.4 show that the large subunit messenger is more rapidly translated in etioplasts and plastids after about 20 hours of light. There appears to be a reduction in LSU translation rate in response to light and this lasts for at least the first 8 hours of illumination. The variation in transit time for ribosomes making this protein is not large (one minute) but it is sufficient to cause a 38 percent decrease in large subunit production.

It may also be significant that LSU messenger transit time responds differently to the illumination of etiolated peas than does the mean plastid ribosome transit time. This could imply differential control of the synthesis of certain plastid proteins. The very slow mean transit time for etioplast ribosomes suggests that many proteins are being synthesised very slowly at this stage of development, although LSU is not one of them. The discrepancy between the two transit time series may reflect differences in the proportion of membrane to soluble protein synthesis during greening. Several authors have reported a far more dramatic increase in membrane compared to soluble proteins in response to light (Grebanier *et al.*, 1979; Cobb and Wellburn, 1973; Grebanier *et al.*, 1978; Mackender, 1978). This can also be seen in Figures 3.10 and 3.12 shown earlier.

3.2.5. *Wheat Chloroplast Protein Synthesis*

In Chapter 1 the suggestion was made that mature etioplasts would rarely occur in nature. However, it was assumed that the formation of a chloroplast from an etioplast would involve biochemical processes analogous to those found during chloroplast development under natural conditions. This assumption can be tested by comparing some of the data just described for plastid development in greening peas with similar measurements made during the development of the second leaf of wheat plants grown in a simulated natural day/night regime.

Protein synthesis in the second wheat leaf was assessed by examining the total polysome profiles (cytoplasmic plus chloroplast)

of the developing leaves (Figure 3.39). Maximum protein synthesising activity appears to occur about 11 days after sowing when the leaf is fully expanded. The highest proportion of ribosomes associated with messenger RNA in polysomal form is found at this time (Table 3.5). Similar results have been obtained elsewhere (Brady and Scott, in preparation).

Days after sowing	Percent ribosomes in polysomes of > 2 ribosomes
9	62.5
10	70.6
11	81.1
12	64.0

Table 3.5. Proportion of wheat leaf ribosomes in polysomes.

These data can be compared with similar measurements for greening pea apices shown in Table 3.1 and Figure 3.4.

As with the peas, there is an apparent peak of protein synthetic activity at or near the end of the leaf expansion phase of growth. As the leaves senesce the polysome profiles decay.

We can see this change specifically for the large subunit of RuBPCase by observing the changes in the size of polysomes making this protein during development by the ferritin-antibody labelling of total wheat leaf polysomes. The histograms in Figure 3.40 illustrate the size of LSU polysomes as the second leaf ages.

A calculation of the mean size of the LSU polysome (Table 3.6) indicates that these polysomes are largest at the earliest times of

Figure 3.39. Total wheat leaf polysomes (the age of the wheat seedlings is indicated) prepared by the method of Davies *et al.* (1972) were separated on isokinetic sucrose gradients. The locations of the 70S, 80S, di-, tri-, tetra-, etc. somes, identified by analytical polyacrylamide gel electrophoresis of recovered RNA, are shown.

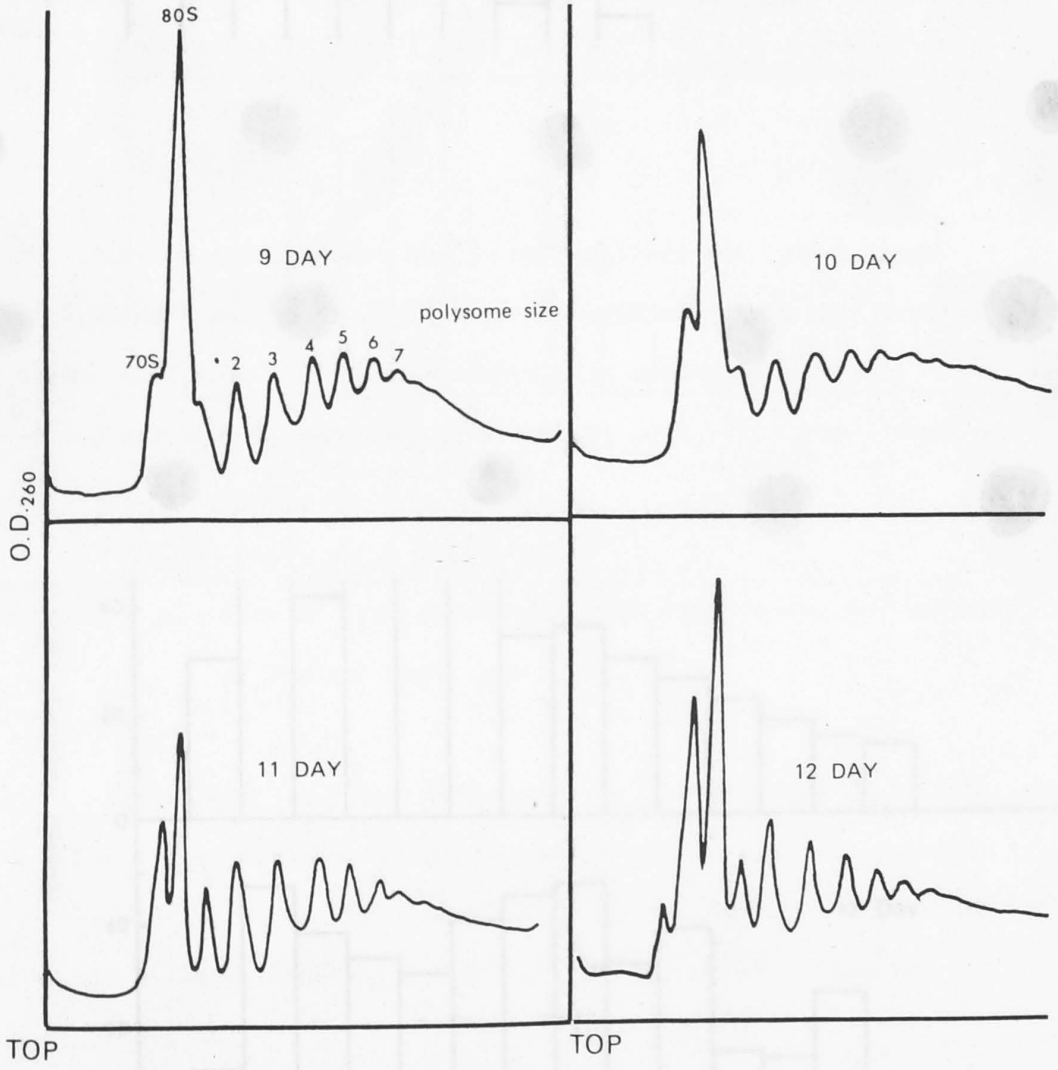


Figure 3.39. Wheat polysome profiles from the developing second leaf

Figure 3.40. The distribution of LSU synthesising polysomes from the second wheat leaf during development (seedlings aged from 9 to 13 days) was determined by labelling polysomes with ferritin-anti-LSU. The location of the chloroplast mono-, di-, tri-, etc. polysomes is indicated across the top of the figure.

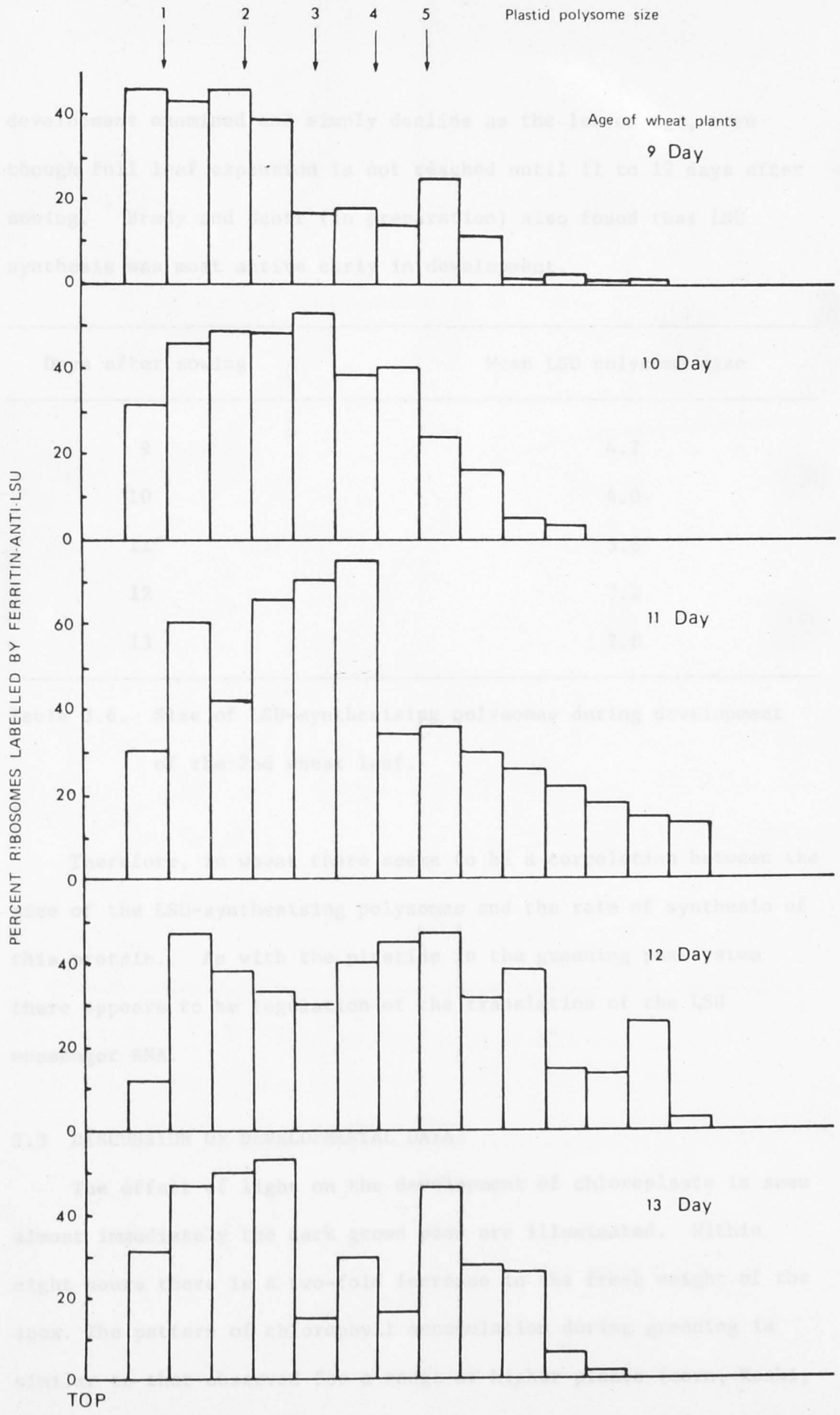


Figure 3.40. Ferritin-anti-LSU labelling of total wheat leaf polysomes

development examined and simply decline as the leaves age, even though full leaf expansion is not reached until 11 to 12 days after sowing. Brady and Scott (in preparation) also found that LSU synthesis was most active early in development.

Days after sowing	Mean LSU polysome size
9	4.2
10	4.0
11	3.6
12	3.2
13	2.8

Table 3.6. Size of LSU-synthesising polysomes during development of the 2nd wheat leaf.

Therefore, in wheat there seems to be a correlation between the size of the LSU-synthesising polysomes and the rate of synthesis of this protein. As with the plastids in the greening pea system there appears to be regulation of the translation of the LSU messenger RNA.

3.3 DISCUSSION OF DEVELOPMENTAL DATA

The effect of light on the development of chloroplasts is seen almost immediately the dark grown peas are illuminated. Within eight hours there is a two-fold increase in the fresh weight of the apex. The pattern of chlorophyll accumulation during greening is similar to that observed for a range of higher plants (corn, Koski,

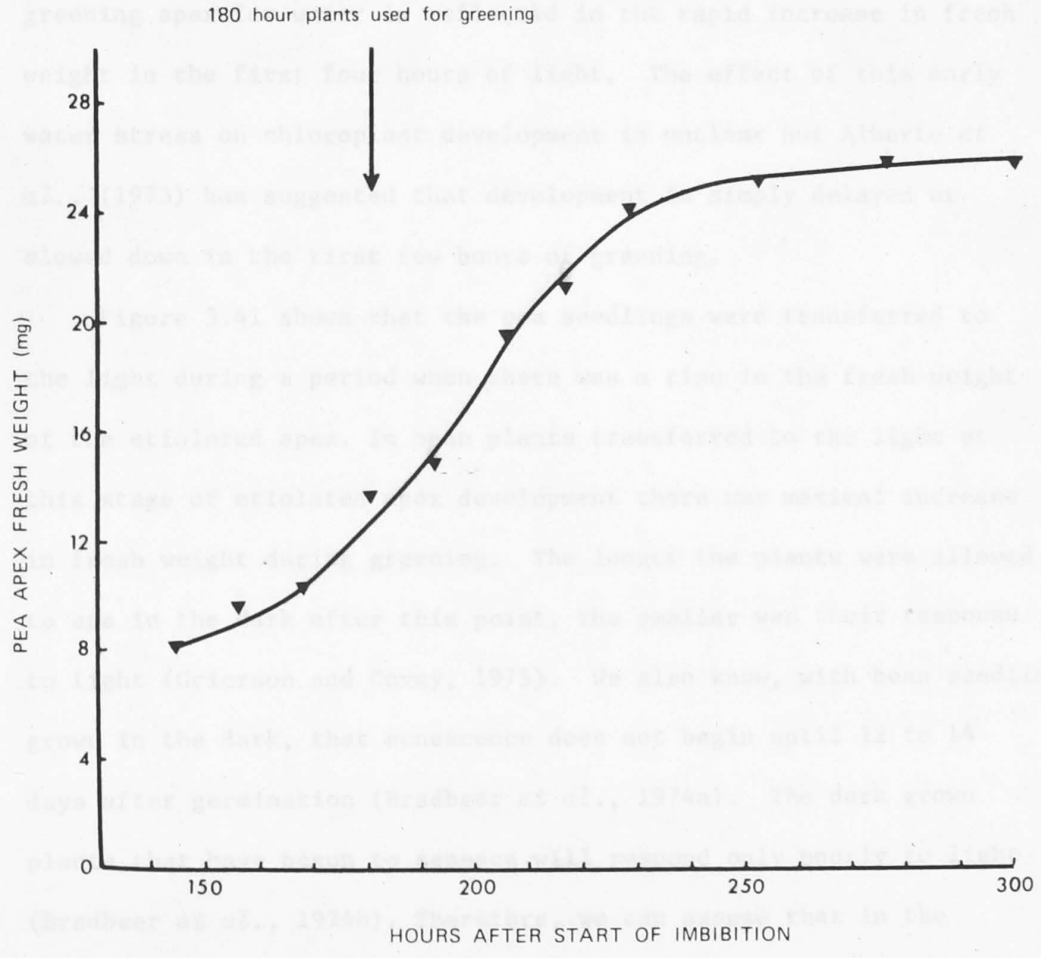


Figure 3.41. The fresh weight of etiolated pea apices

1950; peas, Thorne and Boardman, 1971; bean, Sisler and Klein, 1963; etc). However, there is a lag in chlorophyll accumulation in the early stages of development that is thought to indicate slight water stress (Alberte *et al.*, 1972). The early requirement of the greening apex for water is reflected in the rapid increase in fresh weight in the first four hours of light. The effect of this early water stress on chloroplast development in unclear but Alberte *et al.*, (1973) has suggested that development is simply delayed or slowed down in the first few hours of greening.

Figure 3.41 shows that the pea seedlings were transferred to the light during a period when there was a rise in the fresh weight of the etiolated apex. In bean plants transferred to the light at this stage of etiolated apex development there was maximal increase in fresh weight during greening. The longer the plants were allowed to age in the dark after this point, the smaller was their response to light (Grierson and Covey, 1975). We also know, with bean seedlings grown in the dark, that senescence does not begin until 12 to 14 days after germination (Bradbeer *et al.*, 1974a). The dark grown plants that have begun to senesce will respond only poorly to light (Bradbeer *et al.*, 1974b). Therefore, we can assume that in the experimental system used here the etiolated pea seedlings were capable of showing maximal response to light.

3.3.1. *The proteins of developing pea plastids*

Changes in the synthesis and accumulation of 63 plastid polypeptides during greening are summarised in Table 3.7. The table was compiled with two assumptions in mind; firstly, that each band visible on the stained and fluorographed gel represented a

Polypeptide	Relative Molecular Weight (x 10^3 daltons)	Location of Polypeptide		Accumulation		Synthesis		Nature of Response to Light
		Membrane	Stroma	Period of accumulation (hours in light)	Time of greatest Abundance (hours)	Period of synthesis (hours in light)	Maximum Synthesis Reached (hours)	
1	86	-	+	-	-	0-100	0	-
2	82	-	+	0-100	0	-	-	-
3	76	-	+	0-100	Constant	-	-	x
4	66	+	-	0-8	0	-	-	-
5	62	-	+	0-100	Constant	-	-	x
6	60	+	-	-	-	0-40	8-20	+
7	59	-	+	0-20	0	-	-	-
8	58	-	+	0-100	100	0-100	0-8	+
9		+	-	-	-	0-100	40	+
10	57	-	+	0-8	0	-	-	-
11	55	-	+	0-100	100	0-100	20-40	+
12	52	-	+	0-100	?	0-100	?	+ or x
13	51	-	+	-	-	0-8	0	-
14	50	-	+	0-8	0	-	-	-
15	49	-	+	40-100	100	0-40	20	+
16	48	-	+	-	-	0-40	20	+
17	46	-	+	0-8	0	8-40	20	?
18	45.4	-	+	20-100	100	-	-	+
19	45.2	-	+	-	-	8-40	20	+
20	44.5	-	+	20-100	100	-	-	+
21	43.7	-	+	-	-	20-100	40	+
22	43	-	+	0-40	0	-	-	-
23	40	-	+	0-40	0	0-100	40	?
24		+	-	40-100	100	20-100	40	+
25	39	-	+	-	-	20-40	40	+
26	38	-	+	0-40	0	0-40	20	?
27	37	-	+	-	-	8-100	40	+
28		+	-	0-20	0	8-100	20-40	?
29	35	+	-	40-100	100	0-8	0	?
30	34	-	+	8-100	100	8-100	40	+
31	33	-	+	0-100	Constant	0-40	20	x
32	32.5	-	+	-	-	0-40	0-8	+
33	31.9	-	+	0-100	0	20-40	40	+
34	30	-	+	8-40	20	8-40	20	?
35		+	-	8-100	100	8-40	20	+
36	28	+	-	40-100	100	-	-	+
37	27	-	+	0-40	0	0-40	8-20	?
38	26	+	-	20-100	100	-	-	+
39	25	-	+	-	-	0-40	20	+
40	24	+	-	40-100	100	-	-	+
41		-	+	0-40	0	0-40	8	?
42	23.5	+	-	40-100	100	-	-	+
43	23	-	+	8-100	100	8-40	20	+
44		+	-	40-100	100	-	-	+
45	22	+	-	-	-	8-40	20	+
46	20	+	-	40-100	100	20-40	20-40	+
47		-	+	0-20	Constant	0-40	8-20	x

(Table 3.6. Cont'd.)

Polypeptide	Relative Molecular Weight ($\times 10^3$ daltons)	Location of Polypeptide		Accumulation		Synthesis		Nature of Response to Light
		Membrane	Stroma	Period of accumulation (hours in light)	Time of greatest Abundance (hours)	Period of synthesis (hours in light)	Maximum Synthesis Reached (hours)	
48	17.8	+	-	40-100	100	-	-	+
49	17.6	-	+	0-100	Constant	0-100	20-40	x
50	17.3	-	+	0-100	Constant	8-40	20	+
51	17.2	+	-	40-100	100	8-20	20	+
52	17.0	-	+	40-100	100	0-100	8-20	+
53	16.0	-	+	40-100	100	0-100	8-20	+
54	14.5	-	+	-	-	8-40	20	+
55	14	-	+	0-100	100	-	-	+
56	13.5	-	+	0-100	100	-	-	+
57	11.7	+	-	-	-	8-40	20	+
58	11.6	-	+	-	-	8-40	20	+
59	10.8	-	+	-	-	0-40	20	+
60	10.7	+	-	-	-	8-100	20	+
61	10.4	-	+	-	-	0-40	20	+
62	9.8	+	-	-	-	0-40	20	+
63	9.7	-	+	-	-	0-40	20	+
TOTAL		19	44	43		45		

Table 3.7. The polypeptides of developing pea plastids.

Accumulation - based upon the staining pattern of gels of plastid proteins (for example, Figure 3.10)

Synthesis - based upon the fluorographs of gels of proteins made by isolated pea plastids (for example, Figure 3.12).

Nature of response to light - + increase
- decrease
x no change
? unclear

single polypeptide and secondly, where a stained and labelled band coincided in position, they represented the same polypeptide. Given the large number of bands present in the gels, particularly for the soluble proteins, neither of these assumptions need necessarily be valid but they will be generally true. In some cases the data for protein accumulation contradicts those for protein synthesis. For example, the 37,000 dalton membrane protein is abundant in the etioplast but has been lost after 20 hours in the light, yet a labelled band shows that a polypeptide of the same size is made throughout development with maximum synthesis between 20 and 40 hours. Similar contradictions appear for the 46, 40, 38, 35, 30 and 24 thousand dalton polypeptides. In these cases it seems that the bands identified on the fluorograph do not represent the same protein as appears on the stained gel.

Five reasons can be suggested to account for this contradiction and for bands that may be stained but are apparently not synthesised and *vice versa*.

1. Some of the stained bands may represent polypeptides synthesised in the cytoplasm and transported into the chloroplast. They would not, therefore, be labelled by the isolated plastids; for example, the small subunit of RuBPCase (the 14,000 soluble band).

2. Some polypeptides, labelled or stained, may be contaminants from the cytosol attached to the chloroplast envelope or from other organelles. We know that only 70 percent of the refractile bodies seen in the etioplast preparation, are etioplasts.

3. The specific activity of some proteins may not be high enough for detection or, conversely, the protein may not stain strongly enough to be visible.

4. Some polypeptides are synthesised as precursor molecules which are dependent on cytoplasmically synthesised factors for their processing (Grebanier *et al.*, 1978).

5. Some membrane polypeptides may not be inserted into the membranes immediately after synthesis. We may, therefore, find membrane-destined proteins existing in a labelled form in the soluble plastid phase. Several possible examples of this can be identified in Table 3.7. The 40, 30, 24 and 20 thousand dalton polypeptides all accumulate in the membranes. However, polypeptides of the same apparent size are labelled in the stroma, where they do not accumulate. These polypeptides may be made in the stroma of the plastids and over a period of, perhaps, hours may be inserted into the membranes. A published example is known of the processing and insertion of a chloroplast synthesised protein into the membrane dependent on a cytoplasmic synthesised processing factor (Grebanier *et al.*, 1978).

Some other proteins that may show the behaviour suggested in point 5 above are the 58, 28(27), 17.2, 11.7, 10.8 and 9.8 thousand dalton polypeptides. In other words, over half the membrane polypeptides may fall into this category.

There are also several possible examples of processing; cases where a polypeptide is synthesised by isolated plastids with no exactly corresponding staining band but a stained band nearby that increases during the periods of synthesis of their neighbouring labelled bands. Note, for example, 50,000 and 51,000, the 45,400 and 45,200 and the 44,500 and 43,700 dalton polypeptides.

There are many other similar types of interpretations that can be made from the data in Table 3.7. However, whatever the explanation advanced, the apparent anomalies in the data are evidently explicable in terms of protein movement and modification (cleavage, glycosylation, etc).

The important feature of Table 3.7 is that of the 63 polypeptides tracked through development, a total of 65 percent (84 percent of the membrane and 56 percent of the soluble polypeptides) increase in response to light; only 13 percent (5 percent membrane and 16 percent soluble) were lost during greening. The remaining polypeptides either did not change (8 percent) or could not be accurately evaluated (14 percent). Therefore, one of the effects of transferring plants to the light is to greatly stimulate the apparent amount and diversity of the plastid proteins. Some of the increased protein synthesis is involved in raising the levels of preexisting polypeptides but there are 19 polypeptides, present after 8 to 20 hours of greening but not detected in the etioplast. Conversely, only four polypeptides (66, 57, 50 and 46 thousand daltons) were lost in response to light.

Of the 19 membrane proteins studied during development, 11 (58 percent) were not detectable in the etioplast. Therefore, dark protein synthesis is primarily concerned with the production of soluble proteins, presumably enzymes. Light induction of protein synthesis is most marked for membrane proteins. However, the first new proteins to appear in response to light are located in the stroma of the chloroplast and not in the membranes. For example, the 45.4, 44.5, 34, 30 and 23 thousand dalton polypeptides have all begun to accumulate after only 8 to 20 hours of illumination, whereas the

membrane proteins (for example 40, 35, 28, 26, 24 thousand daltons etc.) do not appear until about 40 hours after the plants have been transferred to the light. There is only a single exception to this, the 30,000 dalton polypeptide, which appears after eight hours in the light.

The major features of plastid protein synthesis during greening can be summarised as follows:

1. A small percentage (9 percent) of proteins disappear during greening. The rate of loss is variable.

2. Most soluble and some membrane proteins are present in the etioplast but have their synthesis and accumulation accelerated in response to light.

3. Over half the membrane and some soluble polypeptides are only synthesised and accumulated in the light.

4. Membrane proteins appear later in the course of greening than soluble proteins.

The general conclusion is that plastid proteins have differential rates of synthesis, accumulation and degradation during greening. This implies that some sort of control exists to regulate the amounts of specific proteins.

3.3.2. *The RNAs of developing pea plastids*

There have been several published reports describing the changes that occur in the RNA content of plastids and of leaves during the greening of dark grown plants (Chapter 1). The data presented here agree well with those published. However, there is little information available on changes in messenger RNA content during plastid development. Experiments described in this chapter show that significant changes occur in plastid messenger activity both on a per plastid basis and for the apex as a whole. There were, however, no apparent changes

in the relative amounts of individual messenger RNAs. The products of *in vitro* translation of messenger RNAs isolated from different developmental stages were essentially identical. It, therefore, seems that changes in messenger level play little role in controlling the differential synthesis of specific polypeptides.

It is worth noting that the synthesis of ribosomal and transfer RNAs responds far more rapidly when plants are transferred to the light than does messenger transcription. Ribosomal RNA per plastid doubles in the first eight hours of illumination, representing a considerable boost in synthesis even though no plastid division occurs during this period. Messenger RNA levels per apex (Figure 3.22) begin to rise significantly only after 20 hours of light.

Although considerable transcription of plastid messenger and ribosomal RNAs occurs during chloroplast formation, it does not seem able to keep pace with plastid division. Therefore, after the first twenty hours of light, when plastids do not divide, plastid RNA declines steadily through to maturity and beyond. However, a continual rise in the plastids' protein synthesising capability occurred until the 20 to 30 hour developmental stage (Figure 3.11). Since the rise in protein synthesis by plastids prior to 20 hours is not explicable in terms of increased RNA levels, it must be due to a greater efficiency of messenger translation. It may also be mRNA efficiency that provides the apparent specificity of translation *in vivo*. At the early stages of development (0 to 20 hours) when many changes were observed in the pattern of proteins made and accumulated in isolated plastids, there was no change in the nature of the *in vitro* translation products. Hence, the changes are not due to transcriptional but, presumably, to translational control.

Before discussing the question of the efficiency of messenger RNA translation by the developing plastids, a comment is needed on the degradation and turnover of chloroplast messenger RNAs. In the next chapter the question of chloroplast messenger RNA stability will be discussed. However, it was noted that with the developing plastids an actual decline occurred in the *in vitro* activity of chloroplast messengers on a per apex basis after 40 hours in the light. This contrasts with the ribosomal RNA which continued to accumulate, though at a reduced rate, to 100 hours. It can, therefore, be concluded that chloroplast messenger RNA degradation does occur *in vivo*.

3.3.3. *Efficiencies of translation in developing pea plastids*

It was suggested in the introduction that translational efficiency was a function of ribosome transit time and the size of the polysomes. In order to account for the data presented for protein synthesis by isolated plastids, it would be necessary to find an increased efficiency of messenger use during the first 30 hours of chloroplast development. During this period *in vitro* messenger activity shows a decline per plastid although plastid protein synthesising activity rises.

The observations of polysome size and transit time strongly imply that messenger efficiency does rise during this developmental period. Unfortunately, it is not possible to make meaningful calculations as to the exact level of stimulation that may result from this increased efficiency of translation of total plastid mRNA. There are two reasons why the data presented cannot be used. Firstly, corrections for changes in the sizes of proteins being synthesised at different developmental times cannot be readily applied to measurements

of the mean ribosome transit time for all plastid proteins (transit time only measures how long it takes for a ribosome to traverse the messenger; it does not take into account the length of the message). The second difficulty in calculating messenger efficiency is the inability to make a direct correlation between messenger activity *in vitro* and number of messenger molecules per plastid. In other words we would need to know messenger levels in absolute rather than relative terms.

The trend, however, is clear. Both the size of the polysomes and the speed with which the ribosomes move along their messengers, greatly increase for the first 40 hours of greening. After this period the polysome sizes tend to decrease. This pattern is particularly strong for the membrane-localised polysomes which nearly double in size during the first 20 hours of illumination. The proportion of polysomes associated with membranes shows a similar response, reaching a peak after 20 hours of light. We would, therefore, expect to see the observed large increase in synthesis of membrane proteins 20 to 40 hours after transfer of plants to the light.

3.3.4. *Efficiency of synthesis of the large subunit of RuBPCase*

The LSU protein was the only polypeptide that could be easily traced throughout development by all the procedures used for assaying protein synthesis. Most importantly, it was the only single protein labelled strongly enough to allow transit time measurements. The abundance of this protein is also of interest since it appears to be reflected in the amount of LSU messenger RNA present in the plastids.

If the radioactivity incorporated into LSU *in vitro* is measured, we find that throughout development it accounts for between five and six percent of the radioactivity in all polypeptides. However, Figure 3.42 shows that LSU synthesis as a proportion of total protein synthesis by isolated plastids varies greatly during chloroplast differentiation. Again there is a conflict between the *in vivo* assays and the *in vitro* translation of pea plastid RNAs. It therefore, appears that translational efficiency is not constant during development or for different polypeptides.

The details of the translation parameters for LSU synthesis are shown in Table 3.8.

Hours in light	0	8	20	40	100
Transit time for LSU-synthesising polysomes (minutes)	1.7	2.7	1.7	2.2	2.3
Rate of LSU chain elongation (amino-acids/second/ribosome)	4.9	3.1	4.9	3.8	3.6
LSU polysome size ribosomes/LSU mRNA	2.8	2.6	3.5	3.3	3.1
Rate of LSU initiation (ribosomes/minute)	1.65	0.96	2.06	1.50	1.35
LSU mRNA efficiency (LSU molecules/mRNA/minute)	1.65	0.96	2.06	1.50	1.35

Table 3.8. Translation of the mRNA for LSU.

Figure 3.42. The radioactivity incorporated into LSU protein by isolated plastids is given as a percentage of total ^{35}S-methionine incorporation into protein during the formation of pea chloroplasts from etioplasts (solid line). The efficiency of the LSU messenger RNA to synthesize protein (estimated from the mean LSU polysome size and the transit time for the LSU synthesizing ribosomes) is also plotted against the developmental stage of the pea apices (broken line).

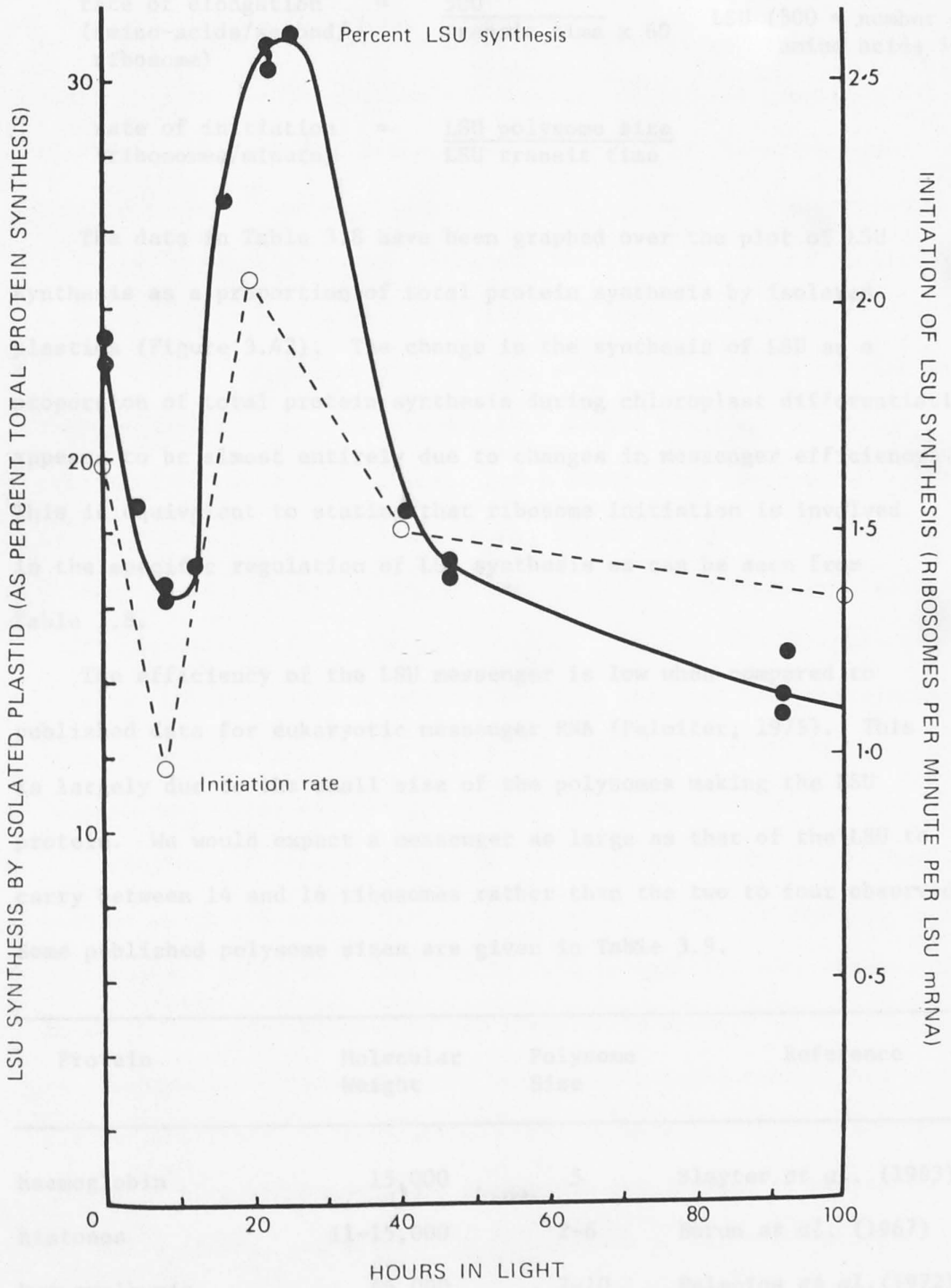


Figure 3.42. LSU synthesis as a proportion of total plastid protein synthesis

$$\text{rate of elongation (amino-acids/second/ribosome)} = \frac{500}{\text{transit time} \times 60} \text{ LSU}$$

(500 = number of amino acids in LSU)

$$\text{rate of initiation (ribosomes/minute)} = \frac{\text{LSU polysome size}}{\text{LSU transit time}}$$

The data in Table 3.8 have been graphed over the plot of LSU synthesis as a proportion of total protein synthesis by isolated plastids (Figure 3.42). The change in the synthesis of LSU as a proportion of total protein synthesis during chloroplast differentiation appears to be almost entirely due to changes in messenger efficiency. This is equivalent to stating that ribosome initiation is involved in the specific regulation of LSU synthesis as can be seen from Table 3.8.

The efficiency of the LSU messenger is low when compared to published data for eukaryotic messenger RNA (Palmiter, 1975). This is largely due to the small size of the polysomes making the LSU protein. We would expect a messenger as large as that of the LSU to carry between 14 and 16 ribosomes rather than the two to four observed. Some published polysome sizes are given in Table 3.9.

Protein	Molecular Weight	Polysome Size	Reference
haemoglobin	15,000	5	Slayter *et al.* (1963)
histones	11-15,000	2-6	Borun *et al.* (1967)
hen ovalbumin	45,000	7-10	Palacios *et al.* (1972) Palmiter (1973)
rat liver albumin	45,000	9-10	Taylor & Schimke (1974)
chich embryo myoglobin	200,000	55-56	Heywood & Nwagwu (1969)

Table 3.9. Published polysome sizes.

Reference to the data in Table 3.9 serves to emphasise the small size of the LSU polysomes. However, Gelvin and Howell (1977) also found that the LSU synthesising polysomes of *Chlamydomonas reinhardii* were unexpectedly small (2-5 ribosomes). The possibility of polysome degradation was discussed earlier. Gelvin and Howell discounted degradation as having affected their result.

The other component of messenger efficiency, rate of polypeptide chain elongation, is also low although within the range of values published for eukaryotic cells; 1.5-8 amino acids per second (e.g., Staehelin *et al.*, 1964; Hunt, Hunter and Munro, 1969; Waldron *et al.*, 1974; Alton and Lodish, 1977; Petersen and McLaughlin, 1973; Scornick, 1974; Alberghina *et al.*, 1975). The elongation rate for LSU is, however, well below those measured for prokaryotes; 5-22 amino acids per second (see Young and Bremer, 1976, for a comprehensive list of elongation rates for prokaryotes).

The low efficiency of the LSU messenger RNA is due to the low rate at which ribosomes initiate synthesis. It is ribosome initiation that appears to be rate limiting for LSU translation and, consequently, it is through initiation that LSU translation appears to be controlled.

Lodish (1976) has suggested that translational control operates, almost invariably, at initiation. Most examples of fluctuation in the rates of polypeptide chain elongation are induced by starvation or other stress, and affect the translation of all polypeptides equally (Forschhammer and Lindahl, 1971; Engbaeh *et al.*, 1973; Boehlke and Friesen, 1975). Some cases where specific proteins are regulated by altered elongation rate have been suggested (Hunt *et al.*, 1969; Orowski and Sypherd, 1978) but the evidence is only indicative of such control. On the other hand there are several

well established examples of translation control mediated through ribosome initiation. These controls have been identified in bacteria (Strome and Young, 1978; Lodish, 1976), fungi (Alton and Lodish, 1978) and mammals (Kurtz *et al.*, 1978; Robbins and Schimke, 1978; McKeehen, 1974). In most of these reported cases of control of translation by initiation the effect is to produce about a two-fold change in the rate of protein synthesis.

There appear to have been few reports of translational control of protein synthesis in plants but the observed effect of ribosome initiation rate on LSU synthesis in peas (also about a two-fold change in rate) is in agreement with the published data for other organisms (references listed above). The mechanism of differential rates of ribosome initiation is not clear, although messenger RNA structure has been implicated (Lodish, 1976).

3.3.5. *Model of Plastid Protein Synthesis during Greening*

In response to light, dark grown pea apices rapidly form functional chloroplasts. Although plastid development in the dark cannot proceed beyond etioplast formation, many of the proteins necessary for chloroplast function are found in the etioplasts. The majority of these are soluble proteins, presumably enzymes involved in CO_2 fixation and in maintaining etioplast metabolism. Most chloroplast membrane proteins are either absent or they are present in only very small amounts in the etioplast. The time course of protein and RNA synthesis during chloroplast development is summarised in Figure 3.43.

In the first 20 hours of greening, the only plastid RNAs that have an increased rate of synthesis are the ribosomal and transfer RNAs. Between 20 and 40 hours after transfer to the light, there is very rapid plastid messenger RNA

Etioplasts	Synthesis of soluble proteins and a few membrane proteins
LIGHT →	
0 hours	
4 hours	Apex fresh weight doubled.
	Synthesis of etioplast specific proteins shut off.
8 hours	Synthesis of new soluble proteins begun; rates of synthesis of others raised.
	Start of rRNA synthesis.
20 hours	Synthesis of new membrane proteins begun; number of ribosomes bound to membranes increased.
	Start of mRNA transcription.
	Start of plastid division.
40 hours	Protein synthesis and transcription at a maximum.
	Time of maximum messenger efficiency.
60 hours	Shut down of transcription and protein synthesis begun.
	Degradation of mRNA.
100 hours	Almost no protein synthesis in old leaves.
	Little mRNA remaining.

Figure 3.43. Time Course of Transcription and Protein Synthesis during Chloroplast Formation from Etioplasts.

synthesis. However, plastid division also begins after 20 hours of light and occurs so rapidly that there is an actual decline in the amount of ribosomal and messenger RNA per plastid in spite of high rates of transcription. Compensating for the declining RNA content of the developing plastids, an increase in the rate of messenger RNA translation occurs. Early in development, plastid protein synthesis appears to be inefficient. The period from 20 to 40 hours after transfer to the light marks the time of most rapid RNA synthesis, protein synthesis and translation rate (as assessed by ribosome transit time). This is also the period when transport and biosynthesis of amino-acids, general metabolic activity and degradation of non-essential proteins is at its height (Kirk and Tilney-Bassett, 1978). The availability of amino-acids and RNAs (ribosomal and transfer) may provide the impetus for raising the rate of translation in the plastids.

In addition to an overall elevated level of protein synthesis during chloroplast formation, there is specific synthesis of certain polypeptides at various rates and times during greening. The synthesis of membrane proteins, for instance, lags behind that of soluble proteins by about 20 hours. This lag may be due to a delay in assembling polysomes on membranes compared to the assembly of soluble polysomes.

Differential rates and times of synthesis of specific proteins are reflected in protein synthesis by isolated plastids and in the proteins made *in vitro* by the *E. coli* cell-free system in response to pea plastid polysomes. This specificity does not reside in the messenger RNA population as assessed by *in vitro* translation.

Therefore, the differential synthesis of chloroplast proteins appears to be due to control at the initiation of translation (the binding of ribosomes to messenger RNA); that is, between transcription and polysome formation. This implies that plastid messenger RNAs differ in their ability to compete for chloroplast ribosomes. Messenger RNA competition has been postulated to account for fluctuations in messenger efficiency in *Xenopus* oocytes (Laskey *et al.*, 1977; Asselbergs *et al.*, 1979) and in anchorage-dependent fibroblast cells (Farmer *et al.*, 1978). There have been no reports of mRNA competition for ribosomes in plants; indeed there have been only a few published reports providing circumstantial evidence for translational controls operating in higher plants (Ho and Varner, 1974 and 1976, Ihle and Dure, 1970, and Verma *et al.*, 1975). The strongest evidence presented here for the existence of translational control during chloroplast formation relates to the synthesis of the LSU protein. The ability of ribosomes to initiate protein synthesis on the LSU mRNA varies by over two-fold during greening.

Based upon the data presented for protein synthesis in pea plastids and for the size distribution of LSU synthesising polysomes in wheat leaves, it seems that the regulation of plastid protein synthesis at the level of translation plays an important role in the overall control of chloroplast gene expression. Transcriptional control during plastid development appears to operate to give the synthesis of ribosomal RNAs or messenger RNA (or both). There is no evidence for discrimination between messenger species. Such discrimination occurs when the plastid ribosomes bind to the initiation site of the messenger molecules.

CHAPTER 4

Fractionation, Properties and Mapping of Some Chloroplast Messenger RNAs

4.1 INTRODUCTION

One of the difficulties associated with an examination of the control of protein synthesis in chloroplasts is in determining the involvement of the plastid genome and protein synthesising machinery in chloroplast development and function. In Chapter 1 it was pointed out that, of the large number of proteins present in the plastids, only two have been conclusively shown to be chloroplast DNA-coded (the large subunit of ribulose-bisphosphate carboxylase and a 32,000 dalton membrane protein) although three or four other proteins are probably so coded.

Most approaches employed to examine the products of plastid DNA directed protein synthesis have failed to give definitive evidence for the coding of particular polypeptides by chloroplast DNA. These approaches include the synthesis of proteins by isolated chloroplasts (e.g., Blair and Ellis, 1973; Bottomley *et al.*, 1974), the translation of chloroplast messenger RNA in a heterologous *in vitro* protein synthesising system (e.g., Hartley *et al.*, 1975; Bottomley *et al.*, 1976; Sagher, Grosfeld and Edelman, 1976), the use of inhibitors specific for plastid or cytoplasmic protein synthesis (e.g., Ireland and Bradbeer, 1971; Gregory and Bradbeer, 1973) and the study of mutants (e.g., Feieraband and Schrader-Reichhardt, 1976). All these techniques depend upon the assumption that the chloroplast mRNAs directing protein synthesis in these systems are coded for by the plastid genome. It is, however, possible that they are nuclear-coded, cytoplasmic messengers that have become associated with the outer chloroplast membrane

(Grebanier *et al.*, 1978; Alscher *et al.*, 1978). The suggestion has also been made that some nuclear-coded messenger RNAs are transported to and translated in the chloroplast (Jennings and Ohad, 1972). These uncertainties can only be overcome if the chloroplast DNA can be shown to play a direct role in coding for the proteins. One technique for achieving this is to use an *E. coli* cell-free extract to transcribe and translate chloroplast DNA and then to identify the products. Bottomley and Whitfeld (1979) found over 20 discrete polypeptide size classes labelled with ^{35}S-methionine when this technique was applied to spinach chloroplast DNA. The major product was identified as the LSU protein but the nature of the other products has still to be determined. An alternative method of locating plastid gene functions is by the hybridization of purified plastid messenger RNAs of known coding function to the DNA molecule. Several other advantages accrue from messenger RNA purification. For instance, the structure of the messenger RNA can be compared with that of its corresponding gene and the nature of transcriptional control elements can be analysed. Radioactive, purified mRNA or a copy DNA to the mRNA can serve as a probe for determining messenger and gene levels within the tissue or tissue extracts. Until the recent advent of DNA cloning and associated techniques, messenger RNA purification appeared to be the only practicable method for achieving such detailed analyses.

However, only two plant messenger RNAs have so far been successfully purified; the messenger for soybean leghaemoglobin (Verma *et al.*, 1974) and the messenger for the maize storage protein, zein (Larkins and Hurkman, 1978; Burr *et al.*, 1978). Amongst the chloroplast messenger RNAs only partial purifications have been achieved. The messenger for the large subunit of RuBPCase was partially purified

from *Chlamydomonas* (Gelvin and Howell, 1977; Howell *et al.*, 1977; Sano *et al.*, 1979) and from *Euglena* (Sagher *et al.*, 1976). The fractionation of this messenger from *C. reinhardii* was dependent upon immunoprecipitation of total cell polysomes, although Howell *et al.*, (1977) added the additional step of first isolating the small polysomes. However, neither group attempted to separate messenger from ribosomal RNA and, as total cell polysome preparations were used, relatively little purification was achieved. Sagher *et al.* (1976) detected the large subunit message in the Poly-A minus fraction of *Euglena* chloroplast RNA and were able to further enrich the message by sucrose gradient fractionation.

Reisfeld *et al.* (1978) succeeded in characterising the messenger RNA for the 32,000 dalton chloroplast membrane protein and achieved its partial purification by sucrose gradient centrifugation. The sizes of these partially purified chloroplast mRNAs are given in Table 4.1.

mRNA coding function and source	Sedimentation coefficient	Molecular weight	Reference
Large subunit of RuBPCase			
Euglena	10-20S	-	Sagher *et al.* (1976)
Chlamydomonas	12-14S	4.5×10^5	Gelvin & Howell (1977) Howell *et al.* (1977)
Spirodela	16S	$5.6\text{-}7.0 \times 10^5$	Reisfeld *et al.* (1978)
Chlamydomonas	19S	7.3×10^5	Sano *et al.* (1979)
32 K dalton protein *Spirodela*	13S	5.0×10^5	Reisfeld *et al.* (1978)

Table 4.1. Published sizes of two chloroplast messenger RNAs.

There are several features of chloroplast messenger RNAs that make their purification difficult. The absence of poly-A from many chloroplast messengers, including that for the large subunit of RuBPCase (Wheeler and Hartley, 1975; Sagher *et al.*, 1976), means that the separation of messenger from ribosomal RNA by the use of oligo-dT or poly-U columns is not possible (e.g., Shapiro and Schimke, 1975; Honjo *et al.*, 1976). In the absence of poly-A tails, it becomes necessary to depend upon other properties unique to the messenger RNA of interest. For example, Greenberg (1976) successfully isolated messenger RNAs lacking poly-A by using Cs_2SO_4 density gradients containing dimethyl sulphoxide. This technique relies on the fact that RNA has a buoyant density of about 1.9 g/cm^3 whereas that of ribosomes is 1.4 to 1.5 g/cm^3. Similarly gradients of the synthetic sugar, metrizamide (Myegaard and Co.), have been used to isolate messengers lacking poly-A from mouse cell cultures using differences in density of messenger ribonucleoprotein particles (mRNP) and ribosomes (Buckingham and Gros, 1975).

Frequently the size of a messenger RNA is sufficiently different from that of ribosomal RNAs and other messengers to make size fractionation a useful purification procedure. Fractionation can be achieved by sucrose gradient centrifugation under native or denaturing conditions (for example, Boedtker *et al.*, 1976; Levy and Aviv, 1976) or by gel electrophoresis. Electrophoresis on polyacrylamide gels (for example Laycock, 1974; Hagen and Young, 1974; Nakin *et al.*, 1976), urea agarose gels (Dudov *et al.*, 1976) or polyacrylamide-agarose (Floyd *et al.*, 1974) gives better resolution than sucrose gradient fractionation but poorer subsequent recovery of the RNA.

In addition to the above techniques that rely on differences in physical properties of the messenger, ribosomal and transfer RNAs to achieve separation, the possibility exists for more specific fractionation of messengers using antibody reactions with individual nascent protein chains on the polysomes. By using this technique many workers have been able to isolate specific polysomes responsible for the synthesis of a single type of polypeptide. The antibodies may be used to precipitate the polysomes either indirectly (Schechter, 1973; Shapiro *et al.*, 1974) or by immunoadsorption (Scott and Wells, 1975; Palacios *et al.*, 1973). Allied with techniques for physical partitioning, immunological identification can provide very good enrichment for a particular message. However, as can be seen from the data in Table 4.2 the most important stage in the enrichment achieved by Jost and Pehling (1976) for the vitellogenin mRNA from chicks relied on the binding of the poly-A tail of the messenger to a poly-U column.

Purification step	Degree of enrichment
Total purified polysomes	1
Heavy polysomes	2.4
Immunoprecipitable polysomes	10
Poly(U)-Sepharose column	1035

Table 4.2. Purification of Vitellogenin mRNA (data from Jost and Pehling, 1976).

In the case of chloroplast messenger RNA fractionation, the techniques available are those dependent upon the physical properties of the messenger and upon the immunological properties of the polysomes.

The abundance of the large subunit of RuBPCase in the chloroplast and its high rate of synthesis *in vivo* and *in vitro* suggested that its messenger RNA might be present in greater amounts than any other mRNA and, therefore, would be an obvious candidate for study.

Although previous attempts to isolate this and other chloroplast mRNAs have not been successful the great use to which these RNAs can be put once purified, seemed sufficient justification for expending further effort on their purification. One of the prime incentives for undertaking this project was the desire to obtain a probe to measure messenger RNA levels during chloroplast development. Although this has not been achieved, and in retrospect was unlikely to provide a messenger of sufficient purity, the experiments were of value in providing further information on the properties of chloroplast messenger RNAs, and their location on the plastid genome.

The fractionation, properties and mapping of spinach chloroplast messenger RNAs will be described. Spinach was used because chloroplasts with a very high protein synthesising activity can be easily prepared from these plants. In addition, as described in Chapter 1 the positions of plastid ribosomal and transfer RNA genes have been located on restriction maps of spinach DNA and can serve as convenient reference sites for the location of other coding regions.

4.2 RESULTS

4.2.1. *Assay of messenger activity and characterisation of products*

In order to monitor the success or otherwise of messenger fractionation procedures, a reliable and simple assay for specific messenger RNAs is needed. The simplest method of assaying messengers is to examine their ability to act as templates for the synthesis of

specific polypeptides in a cell-free translation system. For chloroplast mRNA the most efficient translation system appears to be the *E. coli* cell-free system described by Bottomley *et al.*, (1974), although Sagher *et al.* (1976) were able to translate *Euglena* chloroplast mRNA in a wheat germ cell-free system.

One problem with the *in vitro* translation of mRNAs is the number of labelled polypeptide products that are revealed by SDS-polyacrylamide gel electrophoresis and fluorography. Does each radioactive band displayed on the fluorograph represent the product of a discrete mRNA or are some bands partial products resulting from premature polypeptide chain termination at specific points on the messenger molecule or from partial degradation of the messenger?

If each band is the product of a discrete messenger species then there should be little or no homology between them; conversely if each is the product of the same message then basic similarities should exist. This distinction is important if one is to assess the purity of a messenger preparation on the basis of its translation products. This problem will be considered in relation to the identification of the LSU of RuBPCase as an *in vitro* product of the translation of chloroplast RNA. Bottomley and Whitfeld (1979) demonstrated by partial proteolytic digestion that a principal product of the *E. coli* translation of spinach chloroplast mRNA was the large subunit. Its location on SDS-polyacrylamide gels was, as expected, at 55,000 daltons. However it was not shown whether or not any of the other, lower molecular weight, bands that appeared on the gels comprise partially synthesised large subunit protein.

(i) *Immunological Identification of the Large Subunit of RuBPCase*

Purified antibodies against the large subunit of RuBPCase (raised in rabbits) were coupled via cyanogen bromide to Sepharose 4B. The anti-LSU Sepharose columns were capable of binding up to 200 µg of purified LSU protein per ml of Sepharose. The bound LSU was released from the column by 8 M urea but not by 3 M NaCl. The ability of anti-LSU Sepharose to bind the products of *in vitro* translation of spinach chloroplast RNA is illustrated by Figure 4.1. Virtually all the protein in the translation mix passed through the column or was released by washing in 3 M NaCl; there was no protein detected in the urea-eluted fraction as judged by 280 nm absorbance. However, 21 percent of the radioactivity remained bound to the column. If the translation products of endogenous *E. coli* messenger activity (no chloroplast RNA added to the translation assay) were also passed through the column, 5 percent of the radioactivity was bound. This implies that about five percent of labelled material loaded onto the column will bind non-specifically.

The run-off and urea-elutable fractions from the anti-LSU column (Figure 4.1) were subsequently analysed by SDS-polyacrylamide gel electrophoresis. Figure 4.2 demonstrates that despite the encouraging results in Figure 4.1 there appears to be little specificity of binding to the column. A high proportion of the radioactivity that migrated on the gels in the 55,000 dalton region did not bind (even if passed through the column six times) and a number of other bands did bind. There are four bands that have been totally retained by the column and at least four polypeptides that have not bound at

Figures 4.1 and 4.2. The products of the *in vitro* translation of spinach chloroplast RNA were loaded onto an anti-LSU sepharose 4B column, washed with 3 M NaCl and eluted with 8 M Urea. On Figure 4.1 the solid line shows the distribution of protein in the eluent from the column (absorbance at 280 nm) and the broken line gives the radioactivity in TCA precipitate protein in the column fractions. The fractions "Runoff" and "Urea" were electrophoresed on SDS-polyacrylamide gels to yield the autoradiograph shown in Figure 4.2. The translation products unfractionated by the anti-LSU column, are displayed in the right hand channel.

Figure 4.1. The binding of the *in vitro* translation products of spinach chloroplast RNA to anti-LSU sepharose 4B

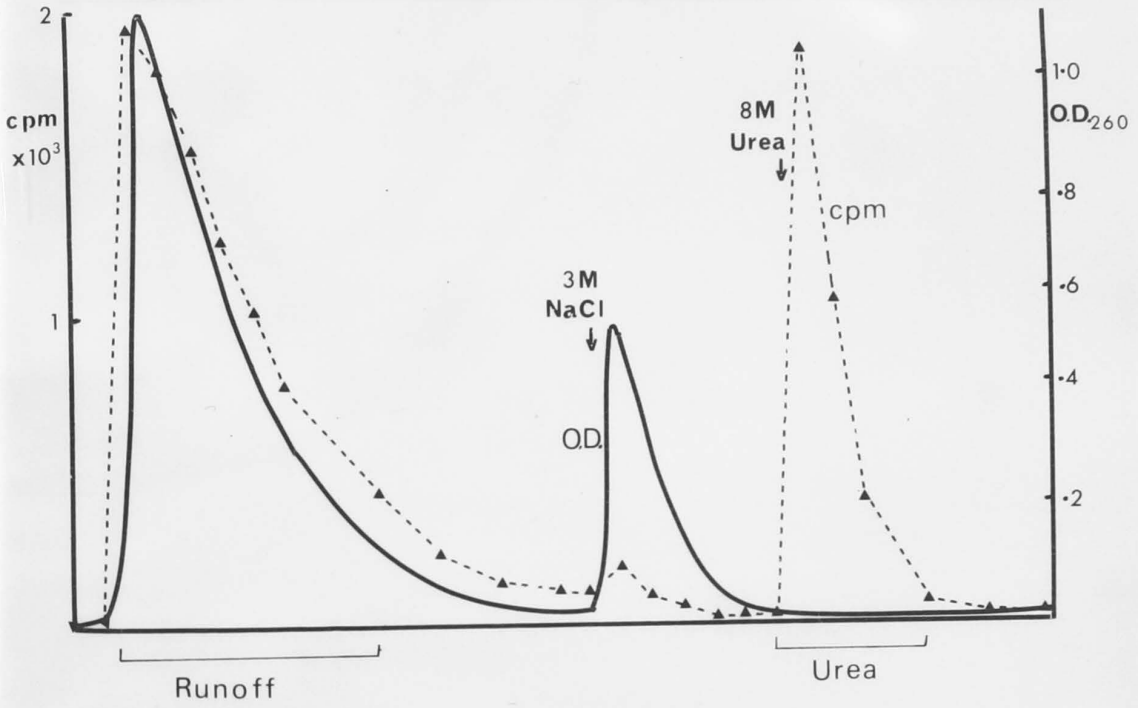
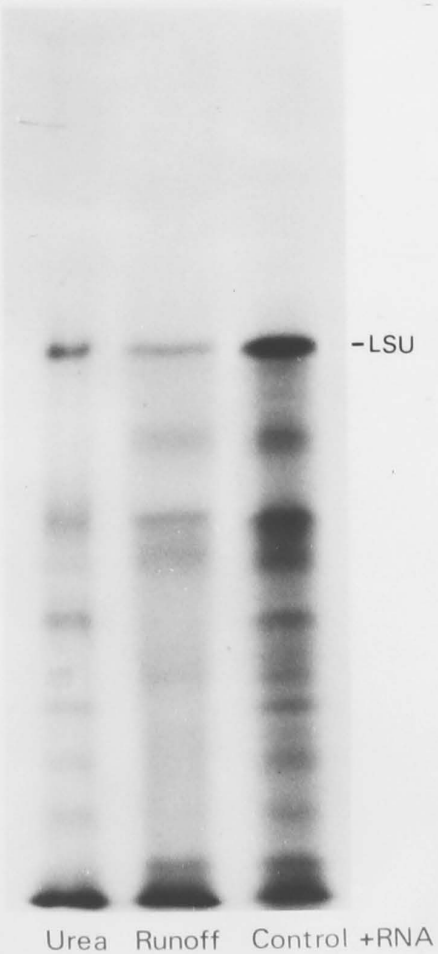


Figure 4.2. *In vitro* translation products fractionated by immunochromatography (Autoradiograph)



all. The column, therefore, appears to show specificity, and quite high specificity, but not necessarily for the large subunit. The possibility exists that there is more than one polypeptide at the 55,000 dalton position on the gels and that the material that does not bind to the antibody column is a polypeptide other than the large subunit. Also the lower molecular weight polypeptides may represent partial products of large subunit synthesis.

In order to test for the degree of similarity between the fraction of *in vitro* (*E. coli* S-30) product bound to the anti-large subunit column and native large subunit, tryptic peptide maps were made. Four fractions were compared with the large subunit protein by tryptic peptide mapping;

1. The *in vitro* product of endogenous messenger activity (no added chloroplast RNA) - "-RNA".
2. The *in vitro* product of spinach chloroplast RNA-directed translation - "+RNA".
3. The fraction of "+RNA" that bound to the anti-LSU column and was eluted with 8 M urea - "Urea".
4. The fraction of "+RNA" that failed to bind - "Runoff".

The resulting "maps" of each of these preparations are shown in Figure 4.3. The diagrammatic representation shows which radioactive spots correspond to ninhydrin-stained large subunit tryptic peptides. If the bound material is large subunit protein, or partial products of its synthesis, one would expect it to show good homology with the ninhydrin stained spots. It does not. Only seven ^{35}S-methionine-labelled spots correspond to stained spots (this is only 23 percent homology). However, the spinach LSU protein contains only 8 to 10 methionine residues (Rutner and Lane, 1967) and consequently, no

Figure 4.3. Tryptic peptide maps were prepared for four samples, the total unfractionated *in vitro* translation products of spinach chloroplast RNA (+RNA), *in vitro* translation products in the absence of added RNA (-RNA), the translation product fraction that did not bind to anti-LSU sepharose 4B (Runoff) and the fraction that did bind (Urea). For each map the ninhydrin stained and radioactivity labelled spots are shown in addition to a line drawing giving both stained (solid lines) and radioactive spots (broken lines). The direction of chromatography (1) and electrophoresis (2) and the origin (O) are indicated.

total
A (+RNA),
translation
nd the

iving
direction
ndicated.

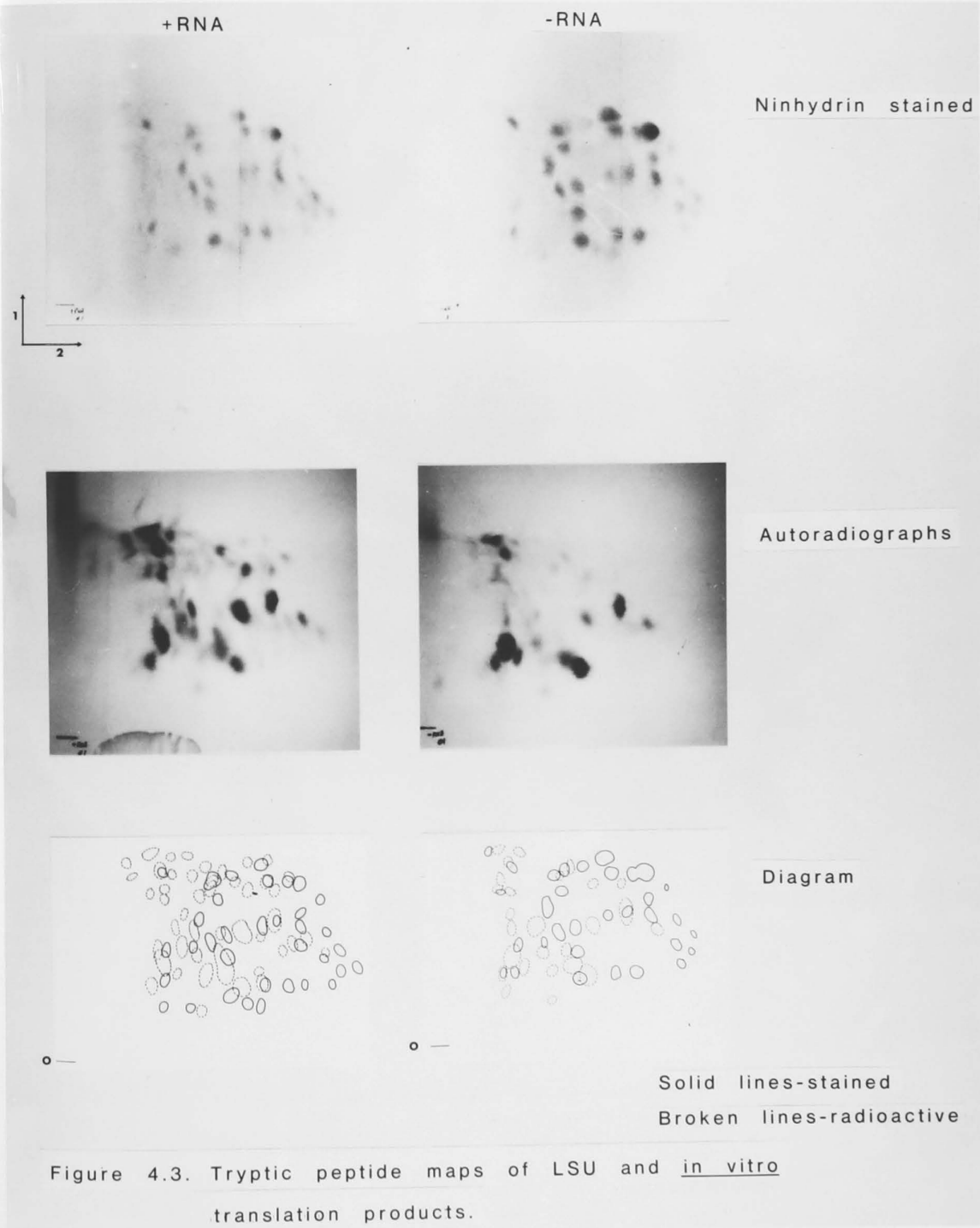
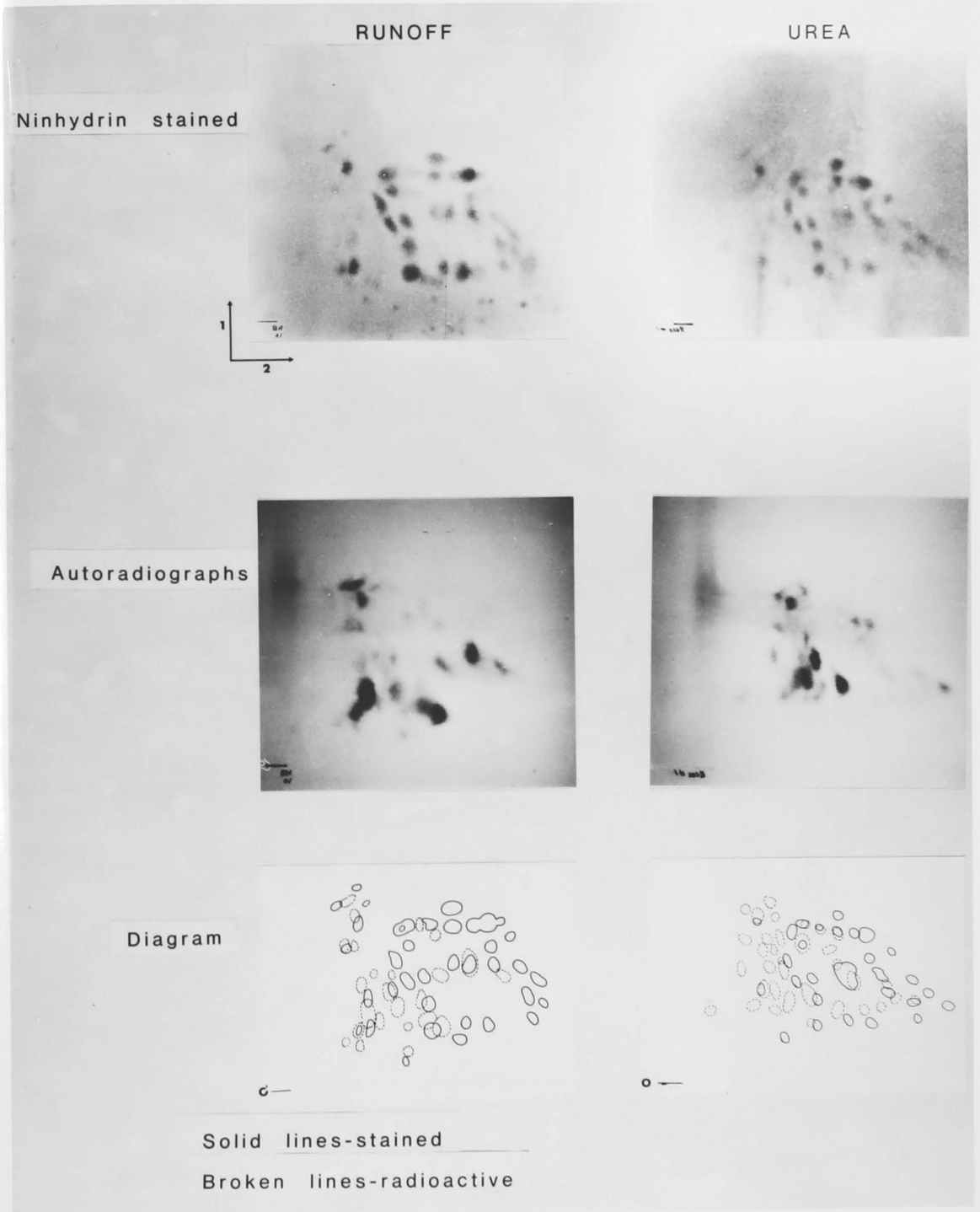


Figure 4.3. Tryptic peptide maps of LSU and in vitro translation products.


RUNOFF
UREA
Ninhydrin stained
1
2
Autoradiographs
Diagram
Solid lines-stained
Broken lines-radioactive

more than 10 stained LSU spots could be labelled with ^{35}S-methionine. The remaining labelled spots may represent tryptic digests of other polypeptides or of modified or incomplete LSU.

The data derived from the peptide maps can be tabulated as shown in Table 4.3. This table shows the relationship for each of the four fractions to each other and to the LSU protein. For example, the second line of the table ("Run-off to Urea") shows that 32 percent of the radioactive spots seen on the tryptic peptide map of the column run-off were also found on the "Urea" map and 31 percent of "Urea" spots were seen on "run-off" map.

Relation	-RNA	+RNA	RUN-OFF	UREA
TO LSU	9	18	8	23
RUN-OFF TO UREA	-	-	32	31
RUN-OFF TO +RNA	-	62	92	-
RUN-OFF TO -RNA	96	-	92	-
UREA TO +RNA	-	30	-	42
UREA TO -RNA	29	-	-	27
+RNA TO -RNA	92	59	-	-
SPOTS UNIQUE TO EACH DIGEST				
-RNA	0	-	-	-
+RNA	-	41	-	-
RUN-OFF	-	-	0	-
UREA	-	-	-	54

Table 4.3 Percent Homology of Tryptic Peptide Digests of ^{35}S-methionine-Labelled *in vitro* Translation Products to native LSU protein.

Points of interest are the following:

1. Greatest homology with LSU is shown by the bound fraction (Urea). This implies some enrichment from 18 to 23 percent homology to LSU.

2. Most labelled spots in the "run-off" correspond to endogenous products (-RNA). 96 percent of the tryptic peptides of the endogenous products appear in the "run-off". This implies specificity for chloroplast-coded polypeptides.

3. Over half the "urea" peptides occur in no other fraction.

No clear conclusion can be drawn from the tryptic peptide mapping. The antibody columns show some specificity but it is not clear what they are specific for. The columns have failed to bind all the 55,000 dalton band from the *in vitro* products, although this band was shown by Bottomley and Whitfeld (1979) and Hartley *et al.* (1975) to be the LSU protein.

(ii) *Evidence that the LSU protein is synthesised* in vitro *via a precursor form*

In the previous chapter it was noted that the major radioactive protein formed by the *in vitro* translation of pea chloroplast RNA appeared to migrate slightly more slowly on SDS-polyacrylamide gels than the equivalent product made by isolated plastids. That result is reproduced in Figure 4.4. It is assumed here, by analogy with evidence from the spinach chloroplast RNA system, that the major radioactive band in the 55,000 dalton region is the LSU protein. An examination of the products formed in chloroplasts when pea apices were labelled *in vivo* with ^{35}S-methionine for one hour, revealed

Figure 4.4. The location of the "LSU", the proposed LSU precursor (P LSU) and the labelling proceedure (*in vivo*, *in organelle* or *in vitro*) are indicated.

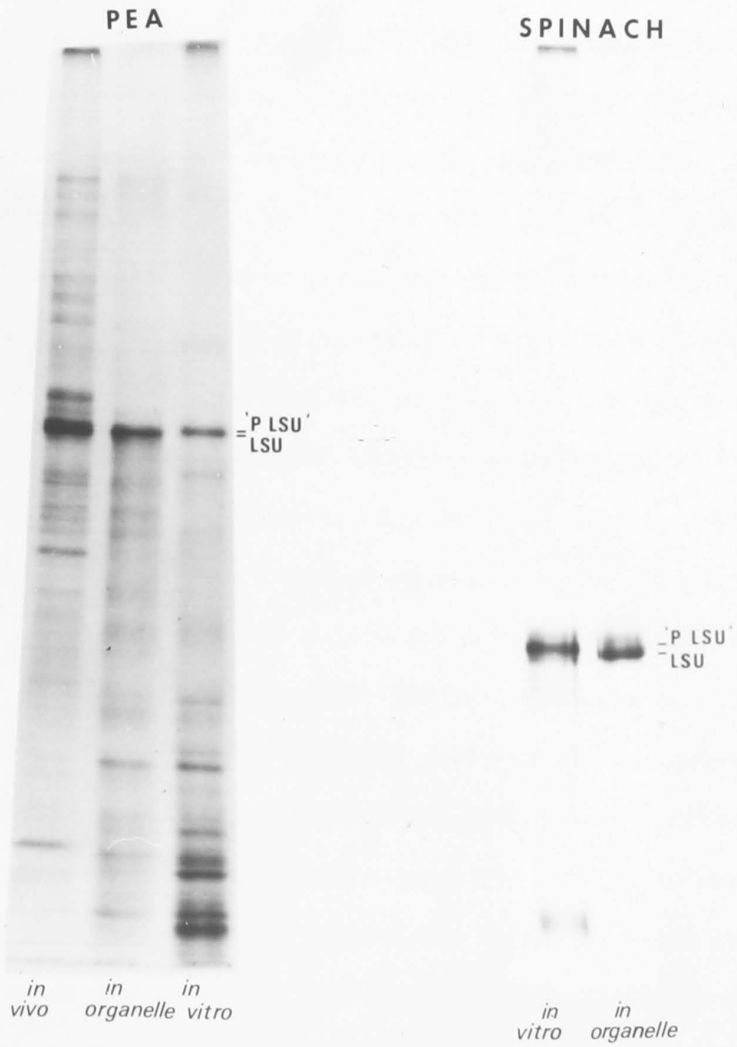


Figure 4.4. Comparison of <u>in organelle</u> and <u>in vitro</u> labelling of pea and spinach LSU. (Fluorographs of gradient gels)

the presence of a radioactive band in the region of the gel coinciding with the stained LSU marker band and also with the product formed by the isolated plastid system (Figure 4.4). Although the difference in mobility between the *in vitro* and *in vivo* products was only small, equivalent to about 1000 to 2000 daltons difference in molecular weight, it was reproducible. By analogy with the synthesis of many other proteins, interpretation of these results is that LSU is synthesised via a slightly larger precursor form. The conditions of labelling used *in vivo* and for the isolated organelle system resulted in the complete processing of the precursor form whereas in the *in vitro* translation system no processing at all occurred.

Further evidence that the synthesis of LSU via a precursor molecule might be a general phenomenon was sought by examining the synthesis of LSU in spinach. The major product of protein synthesis by the *in vitro* translation of spinach chloroplast RNA has been identified as the 55,000 dalton LSU polypeptide (Hartley *et al.*, 1975; Bottomley and Whitfeld, 1979). In Figure 4.4 it can be seen that the ^{35}S-methionine labelled products of the isolated plastid system migrated on an SDS-polyacrylamide gel just ahead of the equivalent *in vitro* translation product. The difference in size between the two forms of spinach LSU is not as great as in the case of the two pea LSU forms (the gradient polyacrylamide gels of spinach proteins were run for twice the time used for pea proteins). In spinach and peas, therefore, it appears that LSU synthesised by isolated chloroplasts is processed to the mature form whereas that synthesised by translation *in vitro* of chloroplast RNA remains as the slightly larger form. The implication of these results is that

there is a processing activity associated with chloroplasts. Unfortunately, the relatively small difference in size of the precursor and final forms makes it a difficult system to study.

4.2.2. *Chloroplast Messenger RNA Fractionation*

(i) *Immunological Techniques.*

The use of antibodies to specifically precipitate LSU-synthesising polysomes was unsuccessful. Techniques of direct and indirect immunoprecipitation precipitated 2 to 3 percent and 10 to 20 percent of the spinach chloroplast polysomes, respectively, but with no apparent specificity. Figure 4.5 shows the translation products of total spinach chloroplast polysomal RNA (channel a) and RNA prepared from polysomes precipitated with rabbit anti-spinach LSU and *Staphylococcus aureus* protein A (channels d, e and f). The three channels shown for the *in vitro* translation products of immunoprecipitated polysomal RNA are: "d", the translation products of polysomal RNA precipitated with anti-LSU only, and "e", "f" the translation products of polysomal RNA precipitated with anti-LSU and two concentrations of protein A. Channels "b" and "c" show the products of endogenous translational activity. There is no apparent difference in the translation products of the control RNA and the RNA prepared by immunoprecipitation. Similar results were obtained using rabbit anti-bean LSU, and sheep anti-spinach LSU. In addition to using protein A as the secondary precipitating agent, goat anti-rabbit, sheep anti-rabbit and rabbit anti-sheep IgG were all used to achieve

Figure 4.5.

Channel	
a	translation products of total spinach chloroplast polysomal RNA
b and c	translation products in the absence of added RNA
d	" " of RNA prepared from anti-LSU precipitated polysomes.
e and f	translation products of RNA prepared from anti-LSU and *Staphylococcus aureus* protein A precipitated polysomes.

The position of the LSU protein is indicated.

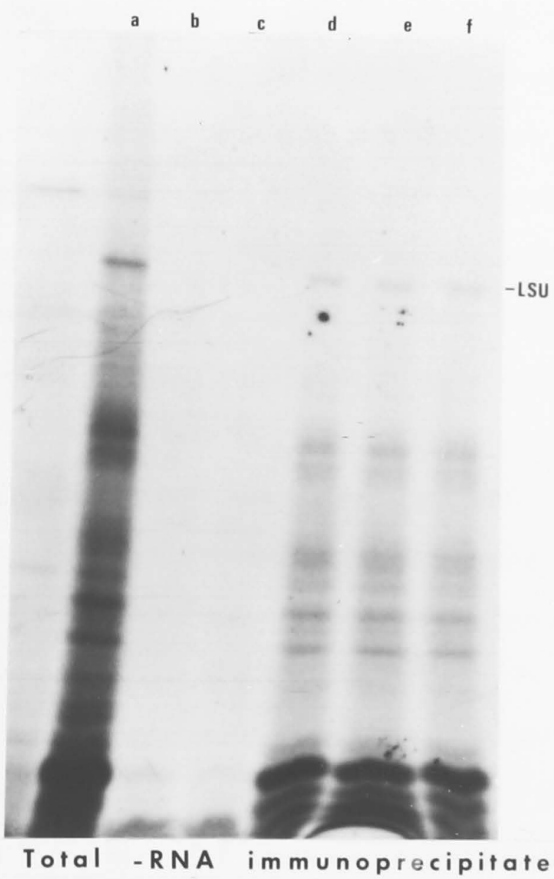


Figure 4.5. The <u>in vitro</u> translation products of spinach chloroplast polysomal RNA fractionated by immunoprecipitation. (Autoradiograph)

indirect precipitation. In each treatment precipitation was still non-specific.

Changes in detergent and salt concentrations used for washing the precipitate did not improve specificity; they simply reduced the yield. Alterations of buffer and salt conditions of the incubation medium also failed to improve the results.

It was also found that the LSU polysomes would not bind specifically to columns prepared with anti-LSU antibody. The support medium initially used for immunoadsorption chromatography was CNBr-activated Sepharose 4B. Since it was thought possible that the CNBr coupling may prevent antibody recognition of the nascent LSU on polysomes, alternative coupling methods were tested. However, m-aminobenzyloxymethyl cellulose (Miles-Yeda Ltd.) and Servacel Cellulose-Ionenaustrausche PAB23 (p-amino benzyl cellulose) also failed to produce columns that would bind LSU polysomes. These two agents link antibody to cellulose by means of diazotization. All columns bound native LSU protein.

The failure of the antibody columns to bind LSU polysomes could be due to failure of the antibody to recognise the nascent LSU protein or to shielding of the antibody or antigen binding site by virtue of the size and shape of the polysomes (ribosomes) and the column media. However, since the ferritin-antibody complex was able to recognise nascent LSU (see Chapter 3), one would expect the LSU polysomes to be recognised by the column-linked antibody. Also the failure of the antibodies to precipitate LSU polysomes directly or indirectly suggests that some factor, other than shielding of binding sites, is preventing the technique from succeeding.

(ii) *Buoyant Density Centrifugation*

In all known cases, messenger RNAs are associated with proteins to form messenger ribonucleoprotein particles (mRNPs) *in vivo*.

The mRNPs tend to contain less protein than ribosomes and hence have a greater buoyant density frequently allowing mRNPs to be separated from the ribosomal subunits on buoyant density gradients. The density of chloroplast ribosomes is about 1.5 g/cm^3 due to a protein content of 46 percent; free RNA has a density of around 1.9 g/cm^3. It is also possible to partially strip off some protein that is not strongly bound to the RNPs by using dimethyl sulphoxide or similar agents. This can further alter RNP densities and induce new differences between various RNP species.

The distribution of chloroplast ribosomal subunits ($O.D._{260}$) and messenger activity (cpm) down a metrizamide buoyant density gradient can be seen in Figure 4.6. The messenger RNP appears to lie under the peak of RNA suggesting that mRNP has not been separated from the ribosomal subunits. An examination of the products of translation by SDS-polyacrylamide gel electrophoresis confirms that there has been no clear fractionation of messengers from each other or from the ribosomes (Figure 4.7, the fraction numbers relate to Fig. 4.7). Therefore, it seems that there is little difference in the percentage protein content of messenger RNP as compared to the ribosomes.

Attempts to partially strip off the protein from RNPs resulted in a complete loss of messenger activity. Dimethyl sulphoxide (DMSO) was used to strip off protein and Cs_2SO_4 provided the density gradient. These gradients gave a single sharp peak of RNA (Figure 4.8), but after RNA isolation no template activity was detected in the *E. coli* translation system. The inactivation was not due to the DMSO treatment but may have occurred during the prolonged gradient run (22 hours at 25°C) or have been due to the procedure used to recover the RNA (see Chapter 2).

Spinach chloroplast ribonucleoprotein was fractionated on metrizamide (Figure 4.6) or CsCl gradients (Figure 4.8). The solid lines give the distribution of RNA (absorbance at 260 nm) and the broken line (Figure 4.7) the *in vitro* template activity of RNA recovered from the gradient. The products of translation of some of the fractions from the metrizamide gradient (fraction numbers on Figure 4.6 and 4.7 correspond) are shown in Figure 4.7. The *in vitro* products of unfractionated RNA and in the absence of added RNA are in the right hand channels.

The buoyant density fractionation of spinach chloroplast ribonucleoprotein

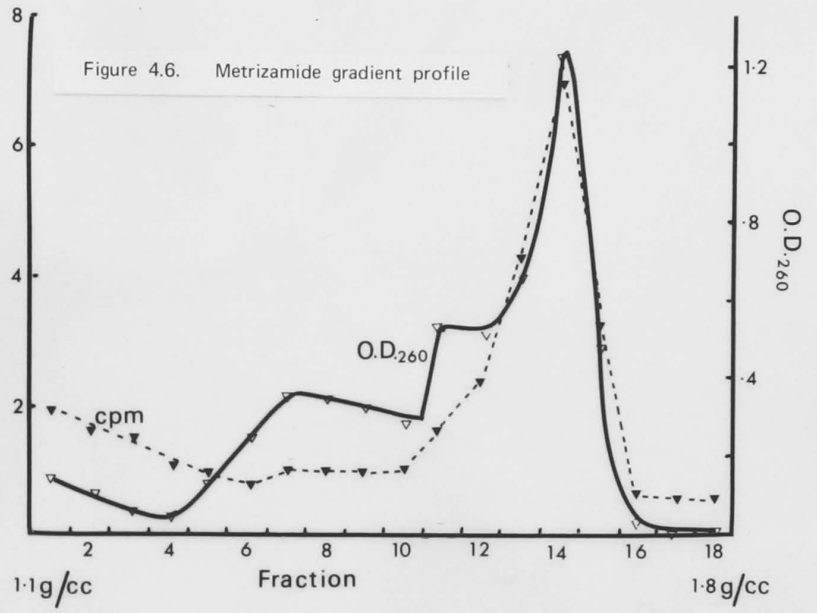


Figure 4.6. Metrizamide gradient profile

Figure 4.7. Products of *in vitro* translation of metrizamide gradient fractions (Fluorograph)

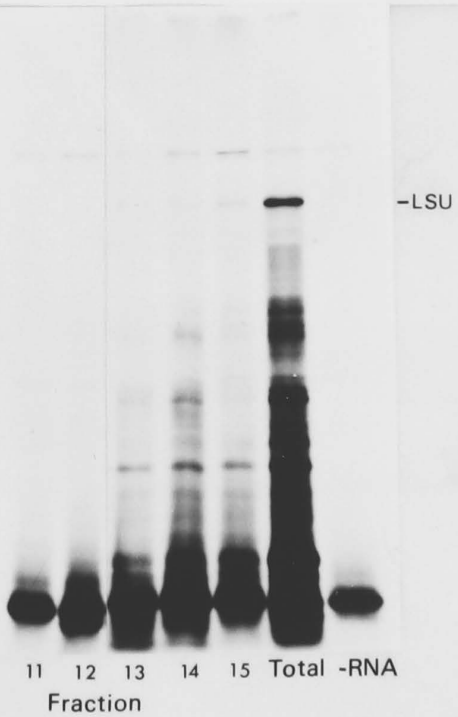
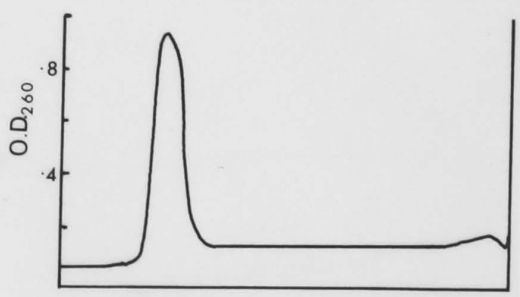


Figure 4.8. Cs_2SO_4 gradient profile



In the case of the metrizamide gradients, over 70 percent of the messenger activity was lost during the experiments although centrifugation was at 8°C. It is possible, therefore, that the high sensitivity of chloroplast messenger RNAs to degradation and inactivation, combined with these long preparative techniques resulted in the loss of activity.

The techniques of buoyant density fractionation gave poor recovery and little or no fractionation.

(iii) *Sucrose Gradient Centrifugation of Chloroplast Polysomes Dissociated with EDTA.*

The previous section described attempts to separate mRNPs from ribosomal subunits on the basis of their buoyant density. It is also possible to separate RNPs from ribosomes on the basis of their sedimentation velocity. Chloroplast ribosomes begin to dissociate into 30S and 50S subunits at Mg^{2+} concentrations below 2 mM. Figure 4.9 shows the effect of low $[Mg^{2+}]$ on spinach chloroplast polysomes when fractionated on sucrose density gradients. In the presence of EDTA dissociation into ribosomal subunits is more complete (see Figure 4.10) and release of the messenger as mRNP also occurs. If the sedimentation velocity of mRNPs differs significantly from those of the ribosomal subunits then fractionation on sucrose density gradients should be possible.

The distribution of messenger activity and ribosomal subunits resulting from the centrifugation of EDTA-treated chloroplast polysomes is shown in Figure 4.10. The peak of messenger activity lies on the light side of the 30S ribosomal subunit. The products of translation of RNA extracted from the gradient fractions are shown in Figure 4.11. The numbers in the fluorograph (Fig. 4.11) correspond to the fraction numbers on Figure 4.10. Although most messenger activity moves more slowly on the sucrose gradient than the 30S subunit,

Figure 4.9. The sucrose gradient profiles were obtained from polysomes suspended in buffer contain 5 mM or 1 mM $MgCl_2$. The peaks labelled on the absorption profile were identified by polyacrylamide gel electrophoresis of RNA recovered from the gradients.

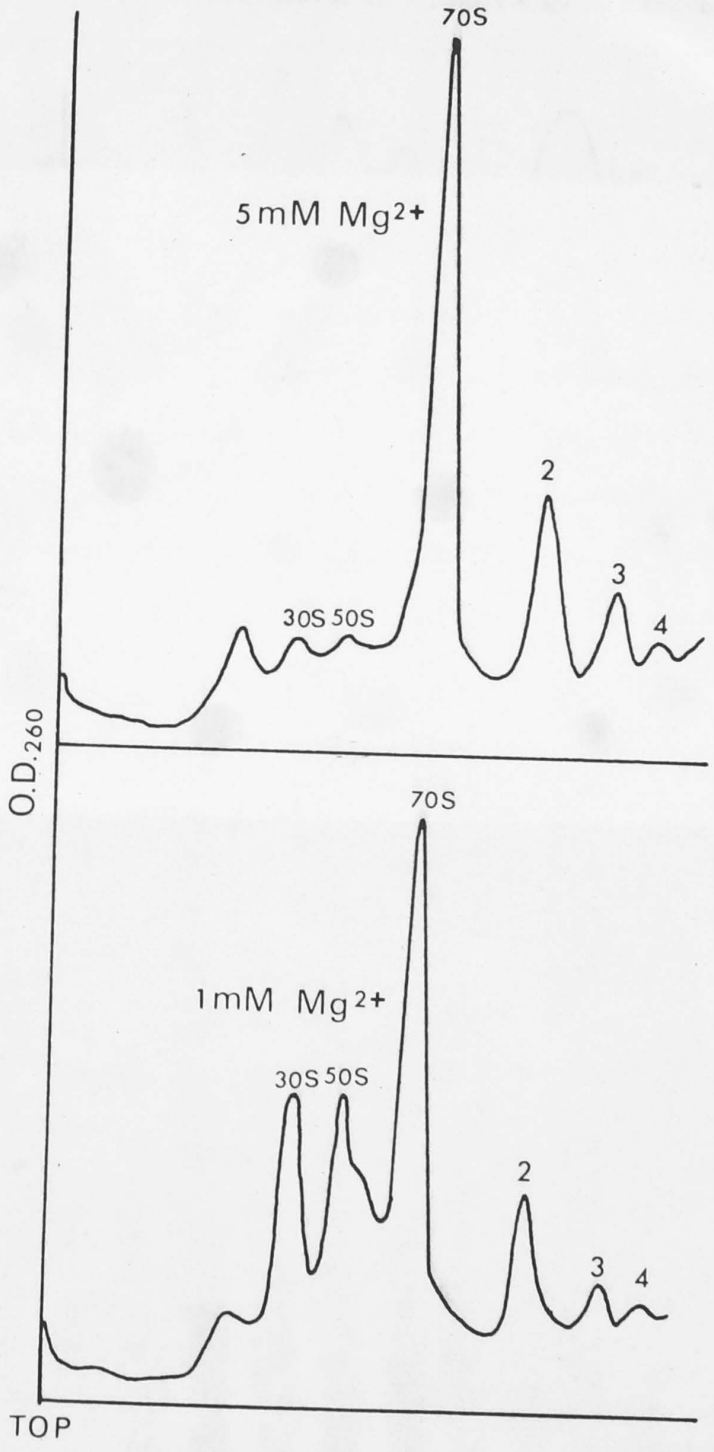


Figure 4.9. The dissociation of spinach chloroplast polysomes in low [Mg^{2+}] (Sucrose gradient profiles)

The absorbance profile (solid line) of sucrose gradient fractionated EDTA-dissociated polysomes and their *in vitro* template activity (broken line) are shown in Figure 4.10. The *in vitro* translation products, corresponding to the gradient fractions, appear in Figure 4.11. The poor quality of the products was due to some difficulties with the assay procedure at the time these experiments were done.

Sucrose gradient fractionation of EDTA dissociated spinach chloroplast polysomes

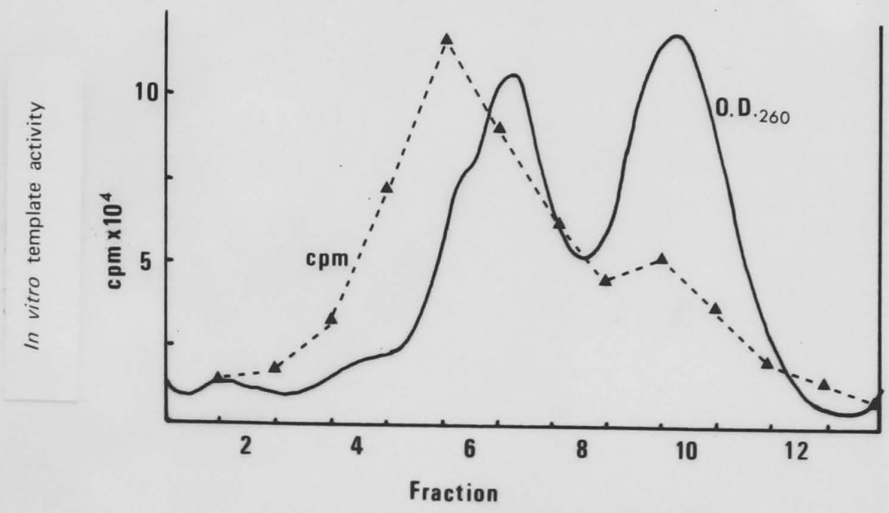


Figure 4.10. Sucrose gradient profile

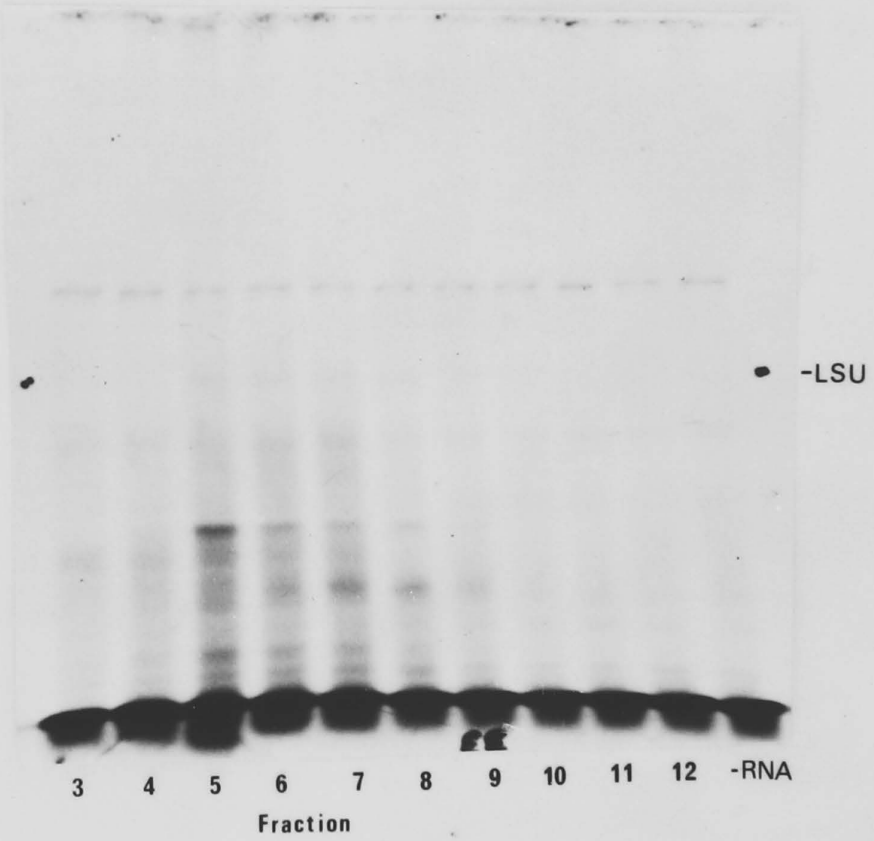


Figure 4.11. *In vitro* translation products (Autoradiograph)

the messenger for LSU (fractions 5-8) is located directly under the 30S peak.

This technique gives good separation of messenger RNA from the 50S ribosomal subunit and consequently from the 23S ribosomal RNA, but it gives little separation of messenger RNA from the 30S subunit and its 16S ribosomal RNA. Unfortunately, only about 30 to 40 percent of the messenger activity of the chloroplast RNA survives the polysome preparation, EDTA treatment, sucrose gradient fractionation and final recovery. The loss of activity is probably due to the fact that only after the sucrose gradient fractionation can the RNA solutions be deproteinised and freed from contaminating ribonuclease. An attempt was made to overcome these difficulties by using "hybrid polysomes".

"Hybrid polysomes" were made by taking a purified RNA preparation from spinach chloroplasts adding it to an *E. coli* cell-free translation assay and incubating the mixture for five to ten minutes so that the *E. coli* ribosomes could bind to the chloroplast messenger RNAs and initiate protein synthesis. At the end of the incubation period, ribosome movement was stopped by adding chloramphenicol to the reaction mixture. The *E. coli*-spinach mRNA polysomes ("hybrid polysome") that had formed during the incubation were collected by centrifugation, followed by EDTA-dissociation of the ribosomal subunits and sucrose gradient fractionation.

It was anticipated that this technique would have two advantages as compared to the direct fractionation of EDTA-treated chloroplast polysomes. Firstly, it used chloroplast RNA that had been freed of protein and was, consequently, relatively free of ribonuclease. The second advantage was that the "hybrid polysomes" contained free

chloroplast messenger RNA (as opposed to messenger ribonucleoprotein) and *E. coli* ribosomes and would, therefore, be free of chloropast 16S and 23S rRNA. Separation of dissociated mRNA from the ribosomal subunits would also be easier as the mRNA was free of associated protein.

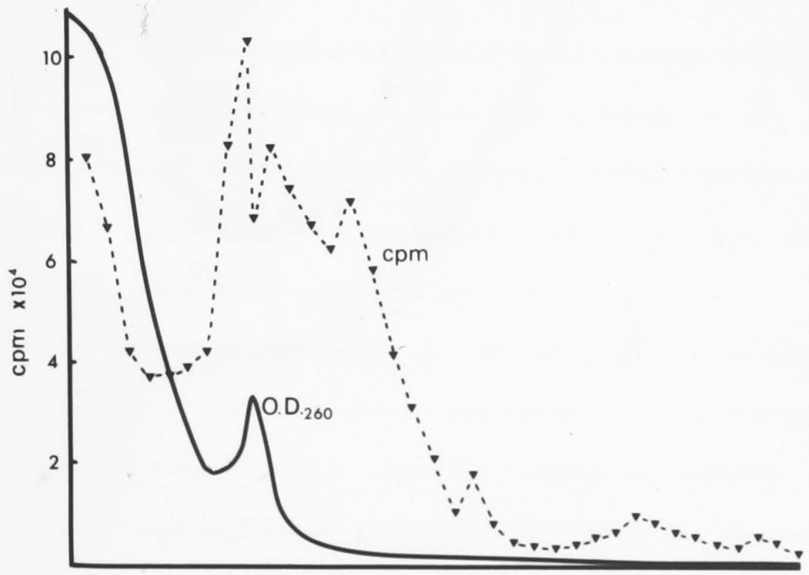
Spinach chloroplast RNA was added to the *E. coli* S-30 assay mix and incubated for ten minutes. The polysomes were collected by centrifugation and loaded onto an isokinetic sucrose polysome gradient. Figure 4.12 shows the distribution of ribosomes and RNA on the gradient ($O.D._{260}$) and the location of ^{35}S-methionine labelled protein (cpm). Most RNA appears to remain at the top of the gradient (the small peak represents the 70S ribosomes) whereas the labelled protein has moved well down the gradient to positions corresponding to mono-, di- and tri-somes. Polyacrylamide gel electrophoresis and fluorography revealed that the labelled proteins were the normal *in vitro* translation products of spinach chloroplast RNA indicating that the polysomes, produced by the assay mix, contained chloroplast mRNA (data not presented). All the messenger appears to become associated with *E. coli* ribosomes since 95 to 100 percent of the messenger activity is recoverable from the ribosomal pellet of the translation assay.

Unfortunately the technique was unusable for the present purposes since all the chloroplast ribosomal RNA cosedimented with the "hybrid polysomes". The cause is uncertain, but the result was an RNA preparation containing all the chloroplast RNA, both messenger and ribosomal, plus the *E. coli* ribosomal RNA from the translation system. The technique preserves messenger activity but provides no useful fractionation.

Figure 4.12. Spinach chloroplast RNA was added to the *E. coli* S-30 assay mix and incubated for 10 minutes. The polysomes were collected and fractionated on an isokinetic sucrose gradient to give the distribution of ribosomes and RNA shown ($O.D._{260}$, solid line). The location of the ^{35}S-methionine labelled protein is also indicated (broken line).

Figure 4.13. Chloroplast RNA was sedimented through an isokinetic sucrose gradient containing 40 mM Tris-HCl, pH 8.5, and 20 mM KCl. The gradient profile and the *in vitro* translation products are shown. The endogenous *E. coli* messenger activity (-RNA) and the products of unfractionated chloroplast RNA (Total) are given in the right hand channels.

Figure 4.12. The binding of *E. coli* ribosomes to spinach chloroplast mRNA *in vitro*



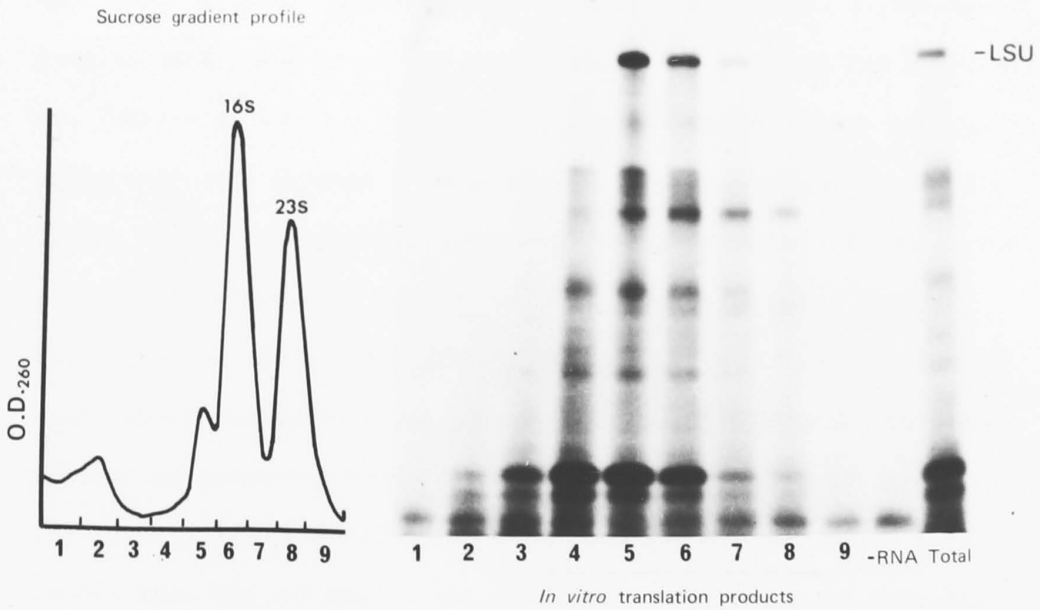


Figure 4.13. Sucrose gradient fractionation of spinach chloroplast RNA in the absence of Mg^{2+}

(iv) *Sucrose Gradient Centrifugation of Spinach Chloroplast RNA.*

Sucrose gradients provide a very simple method for RNA fractionation. Their main advantage is that high recovery of RNA is possible, generally in excess of 80 percent. Their disadvantage is that they provide poor resolution and they are, therefore, most suitable for preliminary purification only.

Maximum resolution on sucrose gradients is achieved if the gradients are isokinetic with respect to the density of particles being fractionated. Appendix 1 describes a method of calculation and construction of isokinetic gradients for any particle of known density. A description of the advantages and properties of isokinetic gradients is also given.

The buffer and ionic conditions are critical in determining the sedimentation characteristics of RNA on gradients. A particular problem with chloroplast RNA is the instability of the 23S ribosomal RNA (Whitfeld, 1978). This RNA usually contains a number of hidden nicks that will produce a range of RNA fragments unless it is held intact by the appropriate ionic conditions. Figure 4.13 shows the profile of chloroplast RNA run on an isokinetic sucrose gradient containing 40 mM Tris-HCl pH 8.5, 20 mM KCl. Note that the 23S RNA peak (which should be twice the size of the 16S RNA peak) is small, indicating extensive breakdown. The degraded 23S RNA has moved under the 16S RNA peak and has also produced the peak sedimenting more slowly than the 16S RNA in the gradients. The messenger activity coincides with the 16S RNA and the 23S breakdown peak. Therefore, on such gradients the messenger fractions contain both 16 and 23S RNAs. The products of the *in vitro E. coli* S-30 translation of unfractionated spinach chloroplast RNA (total) and of endogeneous

E. coli messenger activity (-RNA) are also shown (Figure 4.13).

If 10 mM Mg^{2+} is present in the sucrose gradient buffer spinach chloroplast RNA gives the profile shown in Figure 4.14. In this case the 23S RNA is held intact and the ratio of 23S to 16S is approximately two. However analysis of messenger activity shows that it has resolved into two peaks; one coinciding with the 16S peak (Fraction 5) and one with the 23S ribosomal RNA peak (Fraction 7). The products of the translation of the messenger RNAs are shown in Figure 4.15. From Figure 4.15 it is apparent that the messenger RNA under the 23S peak is produced by aggregation since the products of translation of fraction 7 seem to be identical to those of fraction 5 and also Figure 4.13 showed that under different buffer conditions this heavy mRNA fraction is not present.

The ideal situation, that of no mRNA aggregation and no 23S RNA breakdown, can be achieved by using a gradient buffered with lithium acetate (100 mM, pH 5.0). Figure 4.16 shows the profile of an isokinetic sucrose gradient, so buffered, of spinach chloroplast RNA. Fractions taken from the gradient in Figure 4.16 have been analysed by electrophoresis on urea-agarose gels (Figure 4.17). Messenger activity was located primarily in Fractions 3 and 4. The distribution of messenger RNA can be seen in Figure 4.18 where the products of translation of the fractions are displayed (fraction numbers correspond to the fractions on Figures 4.16 and 4.17).

Greater use can be made of sucrose gradients for fractionation by passing the RNA through two or more cycles of gradient centrifugation (taking fractions from one sucrose gradient for recentifugation through a second gradient). Figure 4.19 shows the re-centrifugation of the 16S RNA fraction recovered from a sucrose gradient fractionation of chloroplast RNA. The RNA was almost totally free of 23S RNA as shown

The gradients used to obtain the RNA fractionation in Figure 4.14 were buffered in 40 mM Tris-HCl, pH 8.5, 20 mM KCl and 10 mM $MgCl_2$. The gradient fractions were translated in the *E. coli* cell-free system and the products are displayed in Figure 4.15. The usual controls of unfractionated RNA (Total) and endogenous activity (-RNA) are given in the right hand channels of the fluorograph.

Sucrose gradient fractionation of spinach chloroplast RNA in 10mM Mg^{2+}

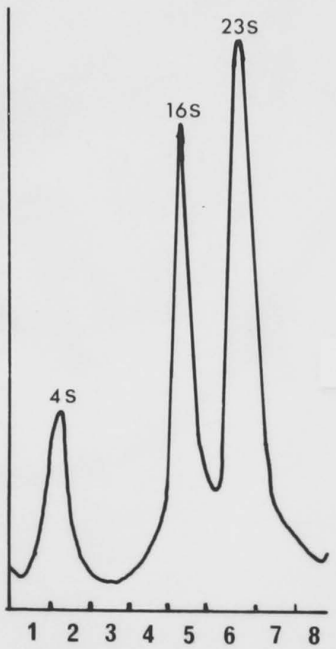


Figure 4.14. Sucrose gradient profile

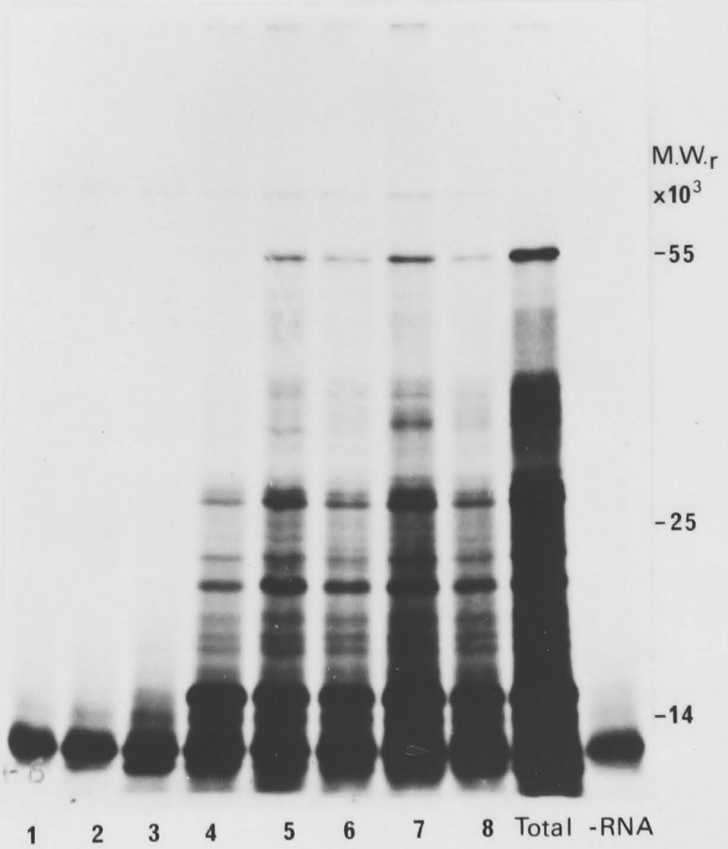


Figure 4.15. *In vitro* translation products (Fluorograph)

The profile of an isokinetic sucrose gradient buffered with lithium acetate (100 mM, pH 5.0) is shown in Figure 4.16. Fractions from this gradient analysed by electrophoresis on urea-agarose gels appear in Figure 4.17 together with unfractionated RNA (Total) and *E. coli* RNA; the position of the 4S, 5S, 16S and 23S RNA species are indicated. Figure 4.18 gives the *in vitro* translation products of the gradient fractions (numbered 1 to 6), unfractionated RNA (Total), and endogenous *E. coli* messenger activity (-RNA).

Fractionation of spinach chloroplast RNA on LiOAc buffered sucrose gradients.

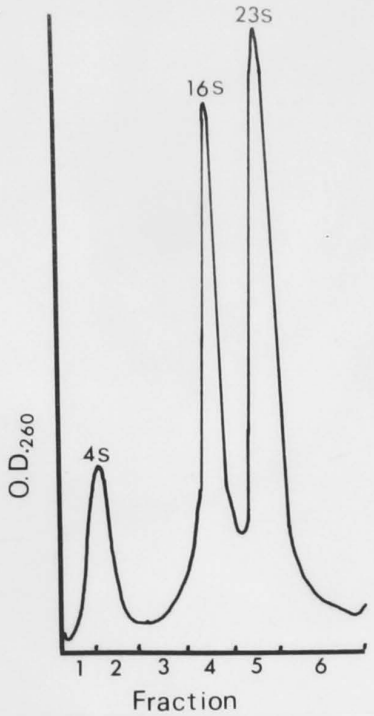


Figure 4.16. Sucrose gradient profile

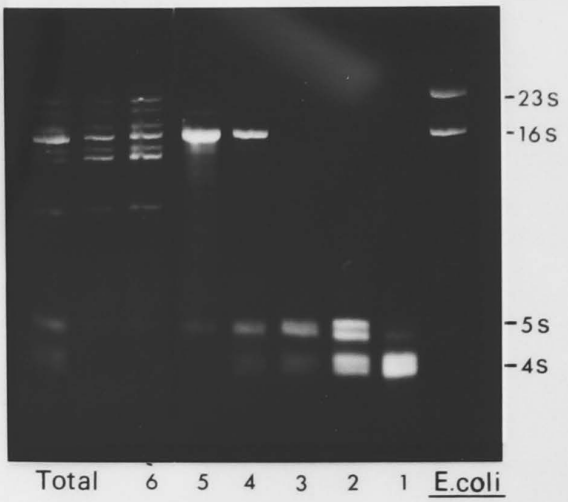


Figure 4.17. Urea-agarose gel analysis of gradient fractions (Ethidium bromide stained)

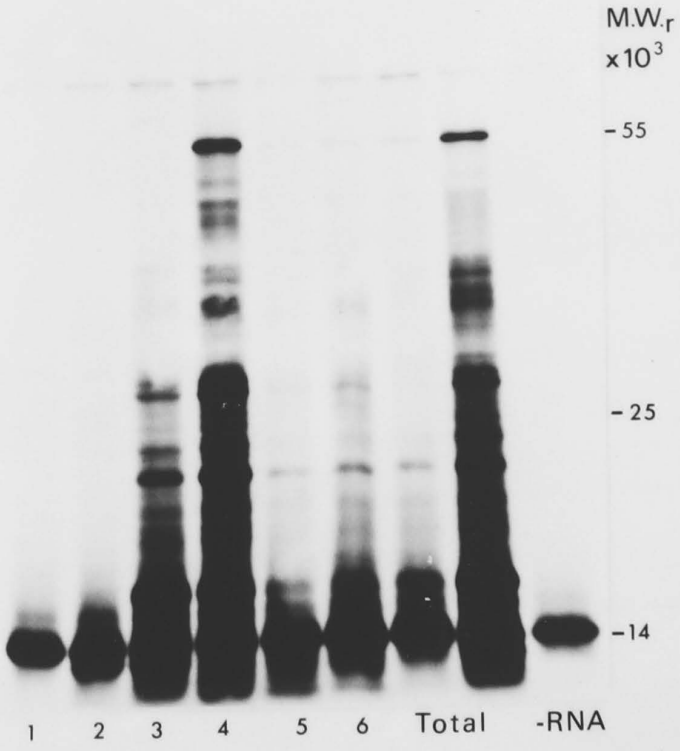


Figure 4.18. *In vitro* translation products (Fluorograph)

by gel electrophoresis (data not presented) but there was little loss of messenger activity. Some removal of 16S RNA from the messenger fractions has also been achieved. The products of translation of the RNAs reveal that there has been some differential sedimentation of messenger RNAs (Figure 4.20). It appears that there are discrete size classes of messenger RNAs in the chloroplast responsible for the synthesis of specific polypeptides. For example, the messenger RNA for LSU appears to sediment at between 14 and 16S (fractions 5 and 6 on Figures 4.19 and 4.20), whereas the mRNA for a polypeptide of about 20,000 daltons has a sedimentation velocity of between 12 and 14S (fractions 3 and 4). However, the possibility still remains that some of the messenger size classes have been produced by the degradation of larger messenger molecules.

(v) *Electrophoresis of RNA on Urea-Agarose Gels*

Gel fractionation of RNA gives far greater resolution than is possible with sucrose gradients. A number of messenger RNA species have been considerably enriched by separation on gels of acrylamide, agarose and a combination of the two media (see 4.1 Introduction). The major problem of gel fractionation has been in recovering the RNA from the gel. In this respect, agarose gels are superior to acrylamide.

In this section the fractionation and extraction of spinach chloroplast messenger RNAs from urea-agarose gels will be described. These gels provide a useful complement to sucrose gradients, the gels fractionating RNA under denaturing conditions and the gradients (used here) under native conditions.

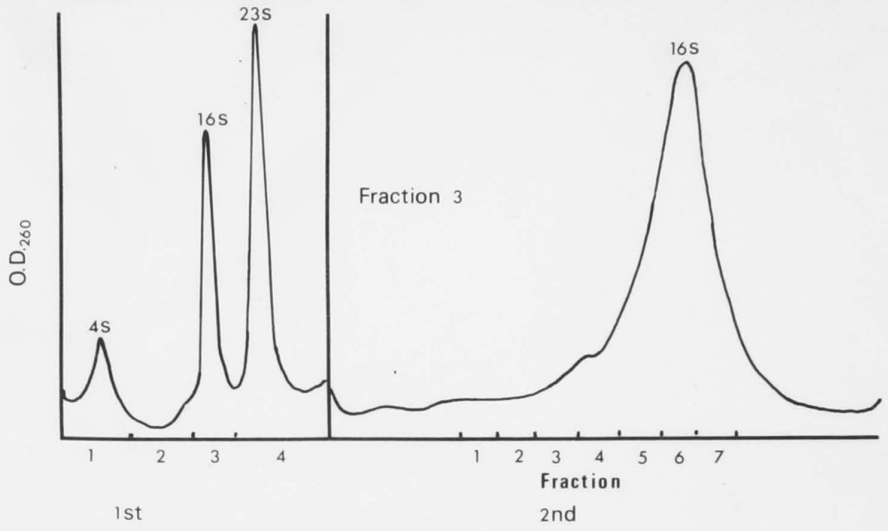
(a) The recovery of RNA from urea-agarose gels

Conventional methods of RNA extraction from agarose gels gave only poor recovery. Only 40-45 percent of spinach chloroplast RNA

Figure 4.19 gives the 260 nm absorption profiles of total chloroplast RNA (1st gradient) and of the 16S RNA (1st gradient fraction 3) recovered from the first gradient (2nd gradient). The translation products of the double fractionated RNA are displayed on the fluorograph (Figure 4.20). The fraction numbers below Figure 4.20 correspond to the fractions of the 2nd gradient and the proteins of the right hand channels were produced by unfractionated RNA (Total) and endogenous *E. coli* mRNA (-RNA).

Double sucrose gradient fractionation of spinach chloroplast RNA.

Figure 4.19 Sucrose gradient profiles



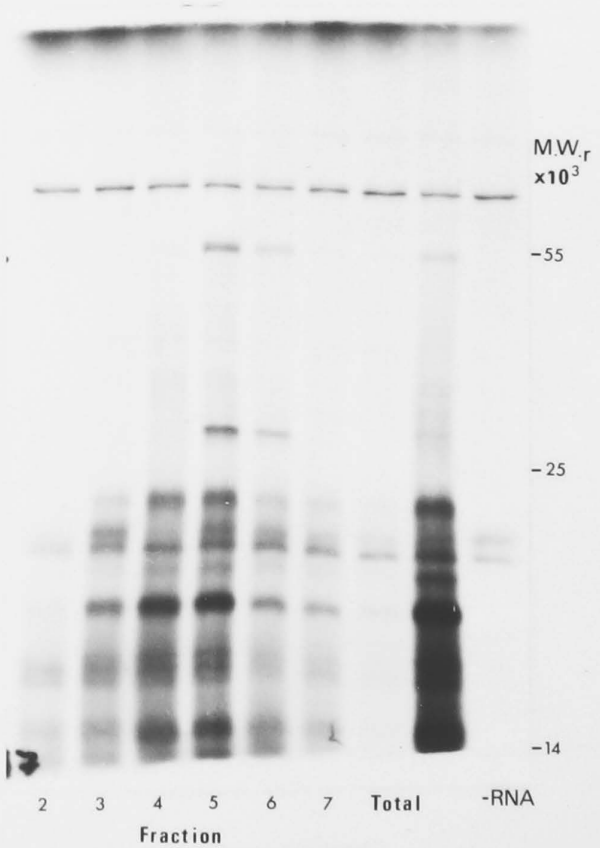


Figure 4.20

In vitro translation products

was recoverable using electroelution. Isolation by the freezing and squeezing (Thuring *et al.*, 1975) or by the diffusion from gels extruded through a syringe gave even lower recovery, 25-30 percent and 20-30 percent respectively. In addition, the RNA solutions obtained by these extraction methods contained dissolved agarose which inhibited their *in vitro* translation. To overcome these problems a procedure was followed based on the experiments of Hurst and Sheng (1976) who used the quaternary ammonium compound, hexadecylpyridinium chloride, to purify glycosamino-glycans. As applied to the separation of nucleic acids from agarose, it is expected that a quaternary ammonium cation (QN^+) will form a complex with the anionic nucleic acid in aqueous solution of low salt concentration, but not with the neutral molecules of agarose. In a two-phase water- alcohol system, the nucleic acid-QN^+ complex would be partitioned to the alcoholic layer by virtue of the hydrophobic hexadecyl side-chain. It may then be transferred to an aqueous solution by raising the salt concentration so that the complex dissociates. The QN^+ compound is then removed by treatment with chloroform.

This procedure requires that both the nucleic acid and the agarose be in solution. This is easily achieved since the urea in the urea-agarose gels lowers the gel melting temperature so that the gel melts after 90 seconds at 75°C and remains molten at 25°C for over an hour.

The efficiency of extraction was determined using *Escherichia coli* ribosomal RNA labelled with [^{3}H]-uridine (300 cpm/µg RNA). A sample of this RNA (100 µg) was mixed with 0.75 ml of molten urea-agarose gel and allowed to set. The gel contained agarose (1.7 percent), urea (6 M) and sodium acetate (0.025 M, pH 5.0) as buffer.

The recovery of RNA after QN^+-butanol extraction, as measured by radioactive counting at various stages, is given in Table 4.4. Similar percentages of final recovery were obtained when the absorbance at 260 nm was used to determine concentrations.

	Percentage distribution of labelled RNA	
	QN^+ 0.67%	QN^+ 3.67%*
In butanol phase after two extractions with QN^+	52	79
In butanol phase after three extractions with QN^+	54	92
Remaining in aqueous phase	46	8
In aqueous phase after two extractions with NaCl	53	92
Remaining in butanol phase	0.7	0.3
Lost to chloroform extraction	0.2	0.2
Final amount precipitated by ethanol	51	92

* Concentration of QN^+ (w/v) added to the equilibrated butanol.

Table 4.4. The distribution of [^{3}H]-uridine labelled ribosomal RNA during extraction with QN^+-butanol from a urea-agarose gel.

The recovery of biologically active RNA by this method was demonstrated by assaying the messenger activity of total chloroplast RNA before and after extraction. Figure 4.21 shows the pattern of peptides synthesised when extracted RNA is used as template in the *E. coli* translation system. RNA not extracted from agarose gels gave the proteins in channels A and B where 140 µg/ml and 360 µg/ml respectively of RNA were used as template. Channels C and D represent the products of protein synthesis directed by RNA extracted from urea-agarose gels using RNA concentrations of 100 µg/ml (C) and

Figure 4.21.

Channel	Concentration of RNA added to the assay	RNA preparation translated
A	140 µg/ml	total untreated RNA
B	360 µg/ml	
C	100 µg/ml	RNA extracted from urea-agarose gel
D	300 µg/ml	
E	0 µg	endogenous *E. coli* mRNA

Figure 4.21. *In vitro* translation products of spinach chloroplast RNA extracted from urea-agarose gels
(Fluorograph)

300 µg/ml (D). The products of endogenous mRNA activity are shown in Channel E. The chloroplast RNA extracted from agarose with QN^+-butanol actually has a template efficiency that is enhanced over that of the starting RNA. It gives both higher absolute levels of incorporation of ^{35}S-methionine and the synthesis of larger polypeptides, indicating less premature chain termination.

(b) Fractionation of chloroplast ribosomal and messenger RNA by electrophoresis on urea-agarose gels.

Spinach chloroplast RNA can be resolved into a large number of bands on 6 M urea-agarose gels, at pH 5.0 (Figure 4.22). The major bands have been identified as the ribosomal and transfer RNAs, including the five breakdown products of 23S RNA. There are, in addition, between 15 and 20 minor bands that stain with ethidium bromide to varying intensities. The major ribosomal and transfer RNA bands are indicated in the figure. The *E. coli* ribosomal RNAs, in the side lanes, show the location of the 23S and 16S RNA species. Figure 4.22 also shows the RNA bands produced by the breakdown of 23S and 16S ribosomal RNAs. Both these RNA fractions were prepared by repeated sucrose gradient fractionation under conditions that held the 23S RNA intact (see earlier). Before loading onto the urea-agarose gel, the RNAs were heated to 75°C for one minute in 10 M urea to denature the RNA. The 23S RNA has been completely broken down. Most of the 16S RNA has remained intact although a large number of degradation products have also been formed from this RNA preparation.

The fractionation of RNA that can be achieved using urea-agarose gels as a preparative procedure is demonstrated by the data in Figure 4.23. Slices were cut from a preparative agarose gel,

Figure 4.22. The type of RNA (described in the text, page 174) used to obtain the patterns shown is indicated below the channels and the amount of RNA loaded is given in μg above each channel. The major RNA species are labelled on the right hand side.

Figure 4.23 A preparative urea-agarose gel was sliced into 18 fractions and the RNA extracted from each slice. The analytical gel shows the major RNA species present in 14 of these fractions and the total unfractionated RNA (extreme right hand channel). The location from which each slice was obtained is given by the gel pattern below.

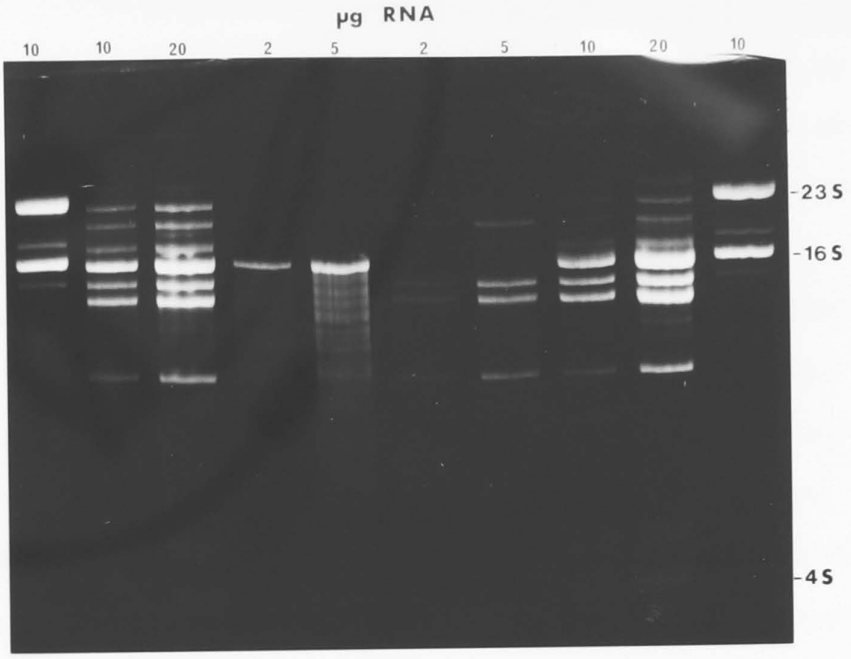


Figure 4.22. Spinach chloroplast RNAs fractionated on a urea-agarose gel. (Ethidium bromide stained)

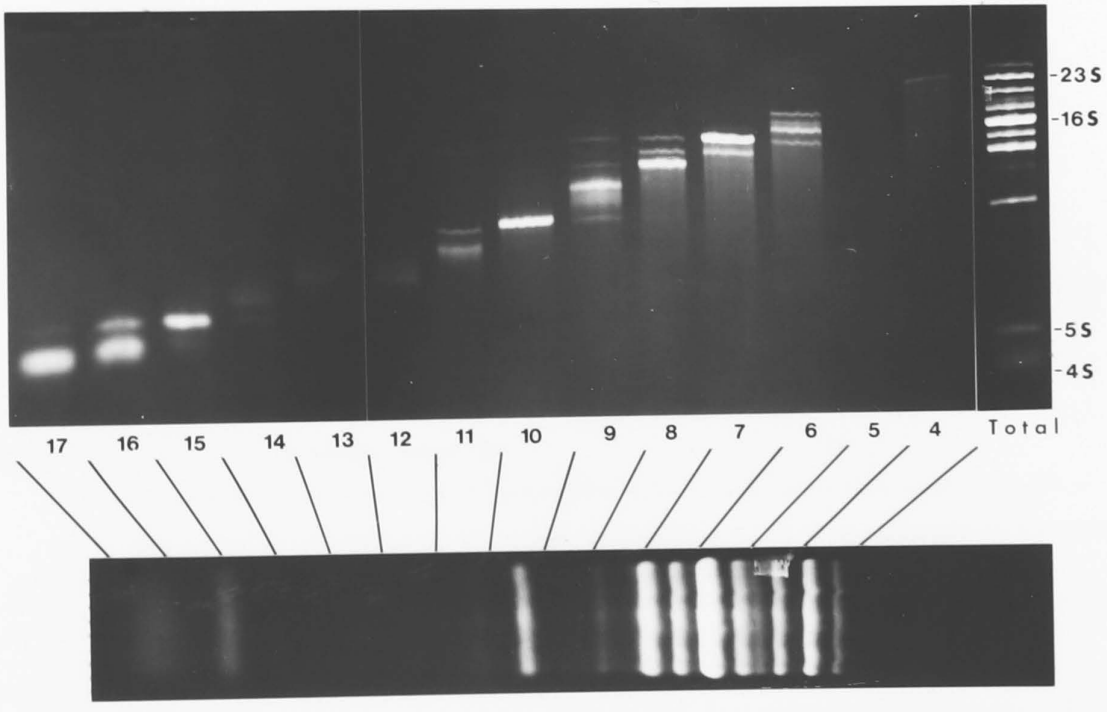


Figure 4.23. Spinach chloroplast RNA fractions extracted from a urea-agarose gel. (Ethidium bromide stained)

the RNA extracted from each slice and re-electrophoresed on an analytical agarose gel (Figure 4.23). The location of each gel slice is indicated on the figure. The technique appears to provide very good enrichment for specific RNA bands.

In the illustrations shown ethidium bromide has been used to stain the RNA bands (Figures 4.22 and 4.23). However, it was found that when RNA stained with ethidium bromide was exposed to ultra-violet light, it was largely inactivated with respect to its *in vitro* messenger activity Table 4.5. For the preparation of messenger RNA fractions, therefore, it was not advisable to stain the gels and it was necessary to cut side-strips off the gels and stain them in order to locate the major RNA bands.

Treatment		Messenger activity (Percent of control)
Unstained	No UV	100
	+ long wave UV	59
	+ short wave UV	44
Stained	No UV	92
	+ long wave UV	38
	+ short wave UV	14

Staining - 15 minutes at room temperature in 1 mg ethidium bromide per 100 ml water.

UV treatments - 5 minutes at room temperature.

Table 4.5. The effect of ethidium bromide and ultra-violet light on spinach chloroplast messenger RNA activity.

The distribution of messenger activity on the agarose gels was determined by *in vitro* translation of the RNA fractions (Figure 4.24). The major chloroplast RNA species were located in Fractions 5 (23S), 8 (16S) and 17 (4S). The messenger RNA for the LSU protein has been localised to a single RNA fraction (Fraction 7) of relative molecular weight larger than the 16S ribosomal RNA (5.6×10^5 daltons). Several other messengers have also been separated from each other and from the ribosomal RNAs. It is interesting to note that the messenger RNA for the 35,000 dalton protein (indicated on Figure 4.24) has moved to a position on the gel close to the 23S ribosomal RNA. This means that the 35,000 dalton polypeptide mRNA has an apparent molecular weight of over 1×10^6 daltons, large enough to code for a protein three to four times that size. A comparison of the mobility of mRNAs on urea-agarose gels and on sucrose gradients is considered in the next section.

A clearer picture of the distribution of chloroplast messenger RNAs can be obtained by a finer fractionation of the gel region between the 1.1×10^6 daltons 23S RNA and the 0.42×10^6 dalton 23S breakdown products. The products of the translation of RNA from this region are shown in Figure 4.25. The ribosomal RNAs were located in Fractions 2 and 3 (23S) and 5 and 6 (16S); the small 23S breakdown fragment of 0.42×10^6 daltons was found in fractions 10 and 11. There are ten polypeptides whose messenger RNAs appear to have been fractionated to some extent. These messenger RNA species can be named after their major translation product (indicated on Figure 4.25 and listed on Table 4.6 in the next section). The possibility, however, exists that some of these messenger species are actually degradation products of one or more of the larger messengers. The messengers responsible for the smaller polypeptides (20.4, 18.6 and 15.5 thousand daltons) show a broad distribution and may represent degraded larger messengers.

RNA fractions recovered from slices of a preparative urea-agarose gel were used as template in the *E. coli* cell-free translation system to give the fluorographs of Figure 4.24 and 4.25. Each figure is labelled on the left hand side to show the position of marker protein and on the right hand side to show the major translation products and their sizes; the numbers below each fluorograph correspond to gel fraction numbers. The right hand channels contain the products of endogenous *E. coli* mRNA (-RNA) and unfractionated chloroplast RNA (Total).

The major chloroplast RNA species were found in the following gel fractions;

RNA	Figure 4.24	Figure 4.25
23S	fraction 5	fractions 2 and 3
16S	" 8	" 5 and 6
4S	" 17	-

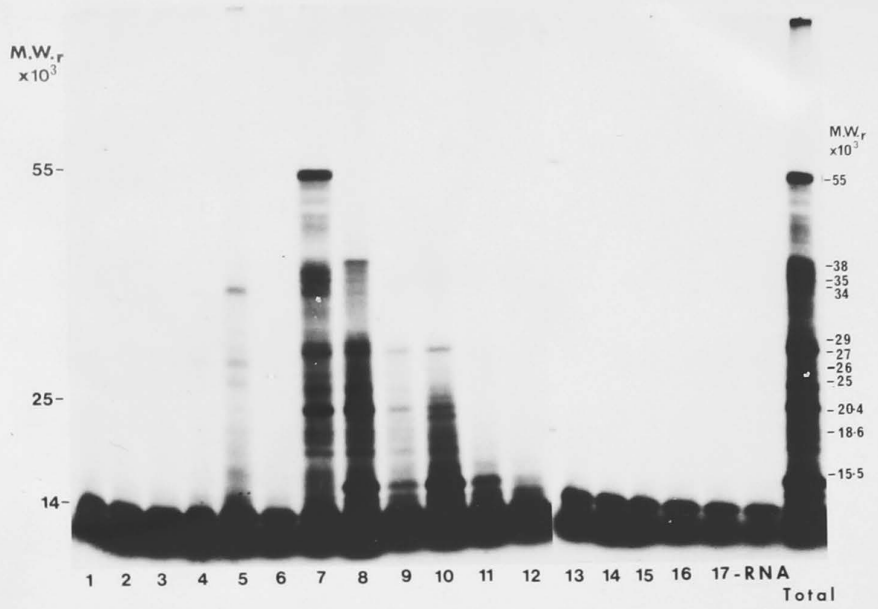


Figure 4.24

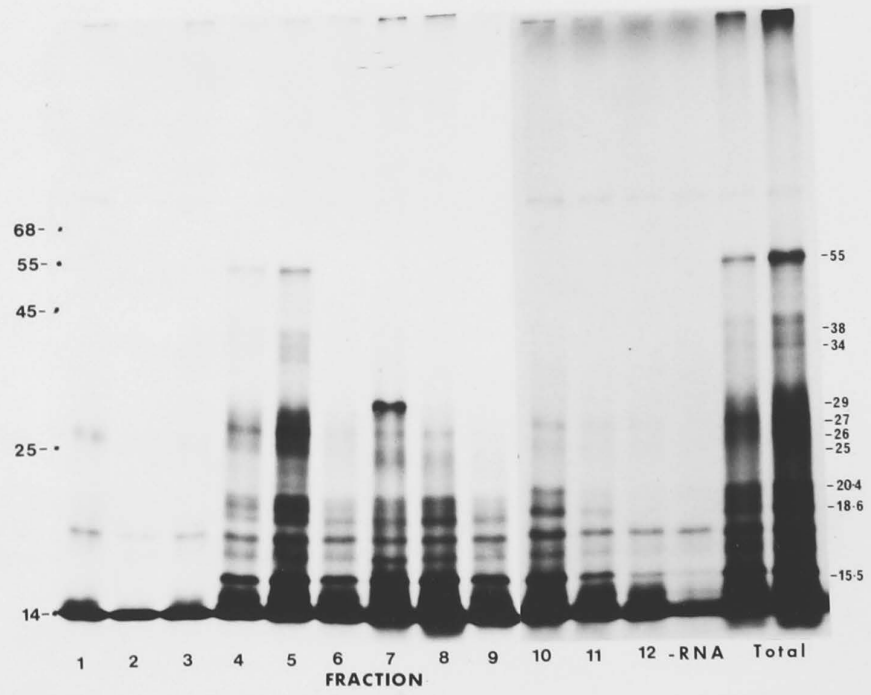


Figure 4.25

In vitro translation products of spinach chloroplast RNA fractionated on urea-agarose gels. (Fluorographs)

The high molecular weight messenger (greater than 1 x 10^6 daltons) has not given rise, in Figure 4.25, to the 35,000 dalton polypeptide seen in Figure 4.24. It has, however, produced a 26,000 dalton polypeptide as a major translation product (this polypeptide is also seen in Figure 4.24). The reason for this difference is not known, but it may result from the presence of more than one mRNA in the fractions, from a multiple coding function of the mRNAs (polycistronic mRNA) or from a peculiarity of the *in vitro* translation system.

(vi) *The Fractionation of Spinach Chloroplast Messenger and Ribosomal RNAs on Sucrose Gradients and Agarose Gels.*

The fractionation and recovery of RNA from sucrose gradients and agarose gels causes only a slight loss of *in vitro* messenger activity (90 percent and 80 percent recoveries, respectively). However, repeated fractionation causes major losses of activity. For example, it has not been possible to recover more than about 10 percent of active messenger RNA from RNA fractionated by two cycles of agarose gel electrophoresis. Sucrose gradients cause less inactivation of the messenger but 10 to 20 percent activity is still lost each time the RNA is run on a gradient. Therefore, multiple fractionation of chloroplast messenger RNAs was restricted to only a single agarose gel fractionation step and no more than two sucrose gradient centrifugations.

The major contaminants of messenger RNA preparations made by agarose gel fractionation are the 16S RNA and the 23S RNA breakdown products. It was considered that if the 23S RNA could be held intact through one or two cycles of sucrose gradient fractionations, an RNA preparation free of 23S RNA and its breakdown products could be obtained from the 10S to 16S region of the gradient where all the mRNA activity was located (see earlier section on sucrose gradients).

The translation products of the RNAs prepared by the agarose gel fractionation of sucrose gradient RNA fractions are shown in Figure 4.26. The nature of the preparative techniques is described on the page facing Figure 4.26. The fluorographs of Figure 4.26 demonstrate the ability of multiple fractionations to provide improved resolution of chloroplast messenger RNAs than is possible by a single step procedure.

Table 4.6 lists the properties of some possible chloroplast messenger RNAs based upon the data shown in Figures 4.25 and 4.26 (the major polypeptide products are marked on the figures) and for the mRNA for the 35,000 dalton polypeptide based upon the data in Figures 4.18 and 4.24.

Major polypeptide product (daltons x 10^3) (marked on Figures 4.24, 4.25 and 4.26)	Messenger RNA size	
	Molecular weight x 10^4 (based on mobility in urea-agarose gels)	Sedimentation (S) (estimated from isokinetic sucrose gradients)
55	75(70-80)	15(14-16)
38	60(60-70)	14(13-15)
35 and 26	110(110-120)	12(11-13)
34	50(40-60)	12(11-13)
29	56(50-60)	12(11-13)
27	46(40-50)	11(10-12)
25	53(40-60)	13(12-14)
20.4	(40-70)	13(12-14)
18.6	(30-60)	12(11-13)
15.5	(30-70)	(11-14)

Table 4.6. Properties of some putative chloroplast messenger RNAs.

Description of the Preparation of RNAs Responsible for the *in vitro* Translation Products Shown in Figure 4.26.

A. Total spinach chloroplast RNA was fractionated on a LiOAc buffered isokinetic sucrose gradient. RNA from the 14S to 18S region was recovered and loaded onto a urea-agarose gel (this fraction was estimated to contain less than 15 percent 23S RNA). Fractions were cut from the agarose gel in the region 7.0×10^5 to 4.0×10^5 daltons. The 16S ribosomal RNA was contained in fractions 2, 3 and 4, and the 4.2×10^5 dalton 23S breakdown was identified, in small amounts, in fractions 7 and 8.

B. RNA was taken from the 10S to 14S region off isokinetic sucrose gradients and loaded onto an agarose gel. The gel was fractionated from the 1.1×10^6 dalton region (Fraction 1) to the 4.2×10^5 dalton region (Fraction 8); the 16S rRNA was contained in fractions 4 and 5. The translation products shown on the extreme right were produced by the sucrose gradient fraction (10S to 14S region) before urea-agarose gel electrophoresis and fractionation.

The *in vitro* translation products of unfractionated spinach chloroplast RNA (total) and of endogenous messenger activity (-RNA) are also shown.

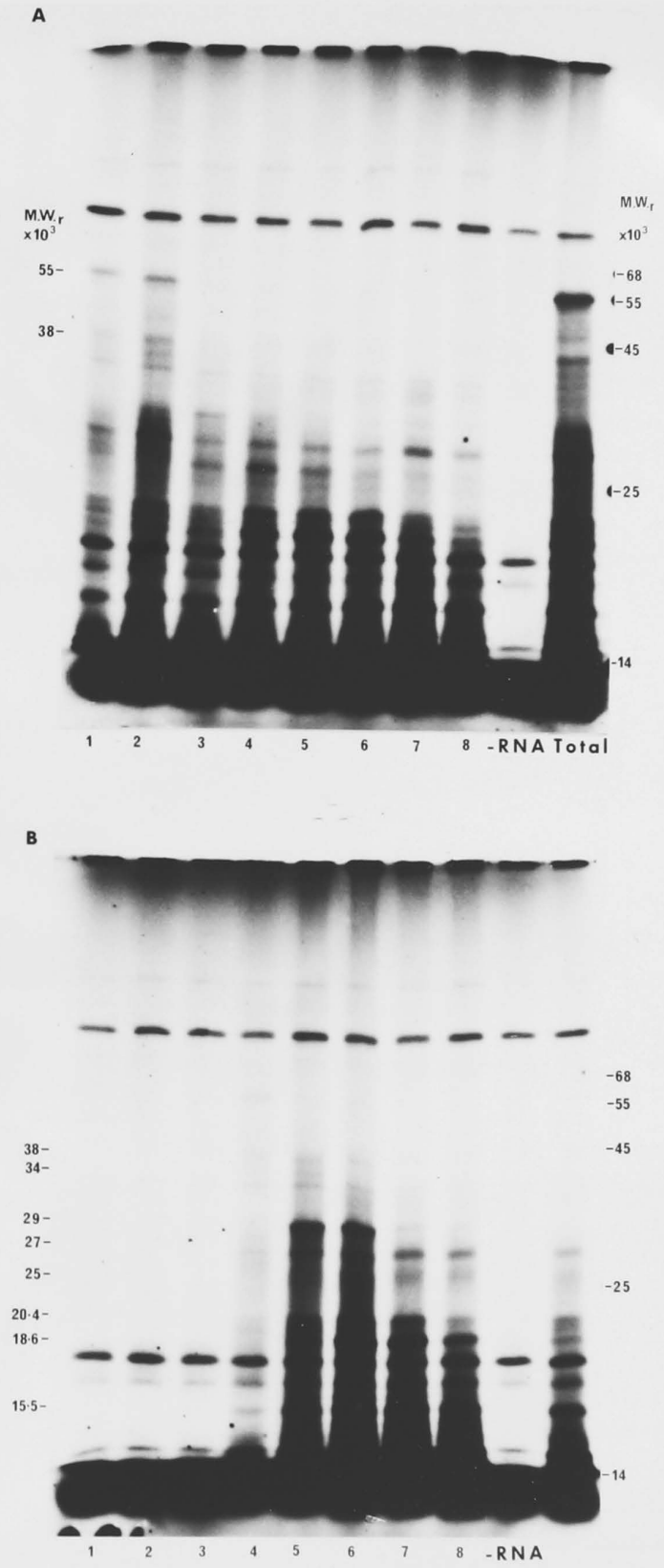


Figure 4.26. In vitro translation products of spinach chloroplast RNA fractionated on sucrose gradients and urea-agarose gels. (Fluorographs)

The data in Table 4.6 provides some information about the hydrodynamic shape of the messenger RNA particles. For example, the messenger RNA that codes for the 35,000 (26,000) dalton polypeptide must exist in a long distended form. This conclusion is based upon the observation that although its molecular weight on urea-agarose gels appears to be similar to that of the 23S rRNA its sedimentation velocity is much lower (12S compared to 23S). The particle must, therefore, have a very high frictional coefficient. All the other mRNA species examined appear to have conformations similar to the ribosomal RNAs since the ratios of their molecular weight to sedimentation velocity are only slightly higher. These conclusions do not apply to the mRNAs *in vivo* where they are associated with protein and probably have a different conformation.

The messenger RNA for the 35,000 (26,000) dalton protein not only has a different hydrodynamic shape compared to the other messengers, but is also the least stable of all the chloroplast mRNAs examined. It was not possible to detect any activity of this messenger after a single sucrose gradient followed by an agarose gel fractionation. This was the only identifiable messenger species that did not survive this fractionation sequence. Perhaps the distended conformation of the messenger molecule renders it more susceptible to degradation than the other messenger species.

4.2.3. *Mapping of messenger RNAs onto chloroplast DNA*

In the preceding sections it has been shown that some fractionation of spinach chloroplast mRNAs on the basis of size differences is possible. It is not known whether the messengers represent the products of transcription of the chloroplast or nuclear genome. Nor is it known whether some of the smaller messenger species are degradation products of larger mRNAs. Both these questions can be answered by mapping the mRNA fractions onto the plastid DNA. If the different RNA preparations map to different regions of the plastid DNA then they not only represent plastid DNA transcription products but also each must represent a discrete messenger species.

The hybridization of four chloroplast messenger RNA preparations labelled with ^{125}Iodine to chloroplast DNA digested with restriction endonucleases is shown in Figure 4.27 (a to d). For each RNA preparation the following illustrations are given:

(i) The *in vitro* translation products of the RNA labelled "A" (fluorograph).

(ii) The fragments produced by the restriction endonuclease digestion of spinach chloroplast DNA (ethidium bromide stained agarose gel; the endonuclease used is indicated).

(iii) Two exposures of the DNA fragments complementary to the ^{125}I-labelled mRNA preparations (autoradiagraph).

Each RNA fraction used for mapping was prepared from total spinach chloroplast RNA separated by electrophoresis on urea-agarose gels. More extensive fractionation of the RNA (such as, sucrose gradient and urea-agarose gel separation) yielded RNAs that gave poor hybridisation to non-ribosomal restriction endonuclease fragments

Figure 4.27. For each RNA preparation (labelled (a) to (d)) the following illustrations are given:

(i) The *in vitro* translation products of the RNA - labelled "A" (fluorograph).

(ii) The fragments produced by the restriction endonuclease digestion of spinach chloroplast DNA (ethidium bromide stained agarose gels). The endonuclease used is indicated.

(iii) Two exposures of the DNA fragments complementary to the ^{125}I-labelled mRNA preparations (autoradiographs).

The RNA preparations were taken from urea-agarose gel fractions of chloroplast RNA as follows:

Figure 4.27 (a) RNA size between 1.3 and 1.1 x 10^6 daltons

(b) " " " 0.75 and 0.65 x 10^6 daltons

(c) " " " 0.60 and 0.50 x 10^6 daltons

(d) " " " 0.45 and 0.35 x 10^6 daltons

Figure 4.27 Restriction endonuclease mapping of spinach chloroplast messenger RNA fractions

Fig. 4.27(a)

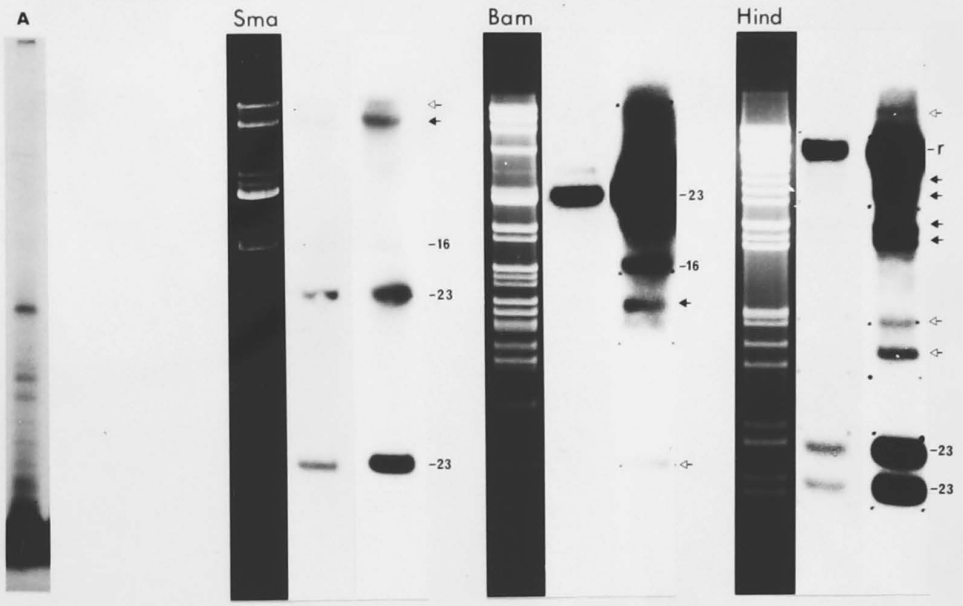


Fig. 4.27(b)

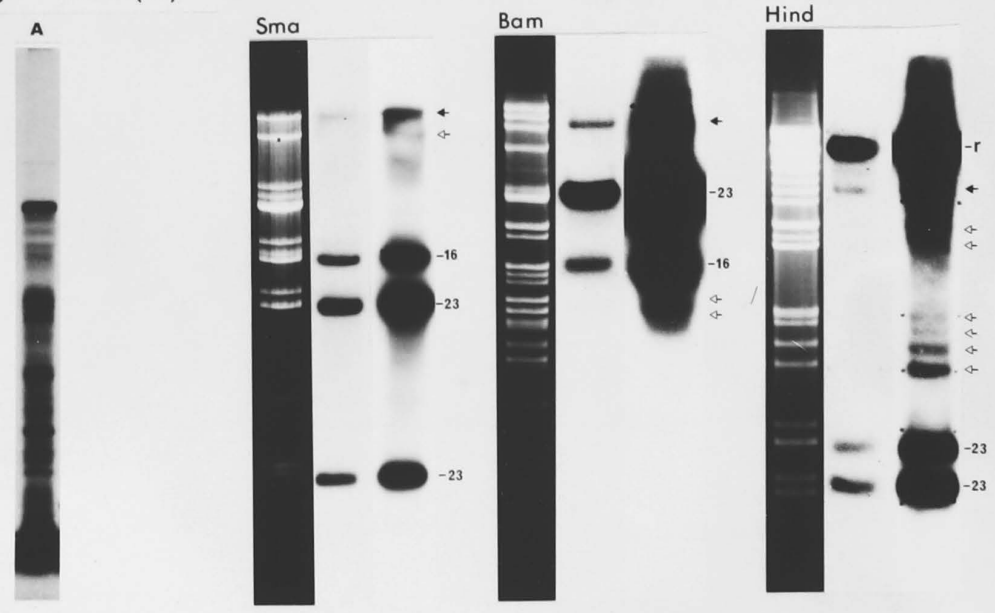


Fig. 4.27(c)

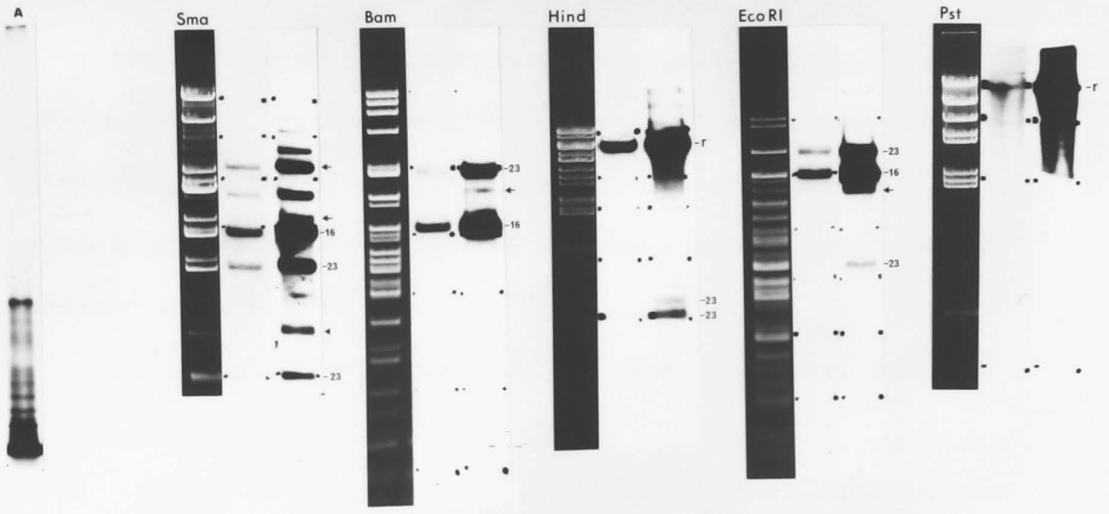
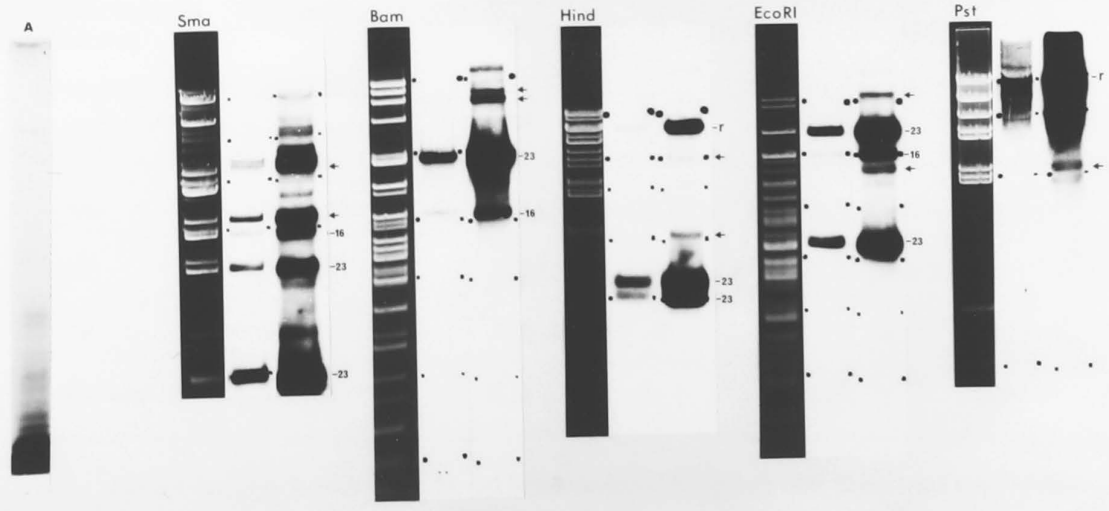


Fig. 4.27(d)



of chloroplast DNA, even though these RNAs possessed substantial *in vitro* translational activity. The fractions taken from the gels were from 1.3 to 1.1 x 10^6 daltons (Fig. 4.27 (a)), from 0.75 to 0.65 x 10^6 daltons (Fig. 4.27 (b)) from 0.60 to 0.50 x 10^6 daltons (Fig. 4.27 (c)) and from 0.45 to 0.35 x 10^6 daltons (Fig. 4.27 (d)).

The RNA preparations were iodinated and hybridised to nitrocellulose filters containing digests of chloroplast DNA (method of Southern, 1975). The DNA fragments containing sequences complementary to the radioactive RNAs were then identified by autoradiography.

The results from Figure 4.27 have been summarised in Table 4.7.

RNA		Restriction endonuclease-digested chloroplast DNA				
Major translation product (daltons x 10^3)	Size of RNA fraction (daltons x 10^4)	Bands labelled, excluding ribosomal genes (in order of intensity) (fragment number)				
		Sma	Bam	Hind	EcoRl	Pst
35	110-120	2(1)	13(22)	9,10,6,7(etc.)	-	-
55	70-80	1(2)	3(13,14)	8(10,11,etc)	-	-
27	50-60	3,6,11	8	-	6	-
25 and 29	40-50	6,3	2,3	14,7	1,6	6

Table 4.7. Restriction endonuclease mapping of chloroplast mRNAs.

The bands referred to in Table 4.7 are indicated on Figure 4.27 by arrows. The solid arrows represent major bands showing hybridization to the labelled RNA and the open arrows minor bands. These minor bands are shown in brackets in Table 4.7. The locations of the ribosomal genes (shown in Figure 4.27) are based upon the results of Whitfeld

et al. (1978) for Sma, Pst and EcoR1 and of Bohnert *et al.* (1978) for Hind III and Bam (the bands labelled "r" contain both 23S and 16S rRNA genes).

Although the RNA preparations show most hybridisation to the ribosomal genes, each of the messenger RNA preparations has also hybridised to other regions of the chloroplast genome. In the case of the messenger RNA for the LSU protein, the spinach chloroplast restriction fragments labelled with ^{125}I-mRNA (LSU) correspond to those labelled by cloned *Zea mays* chloroplast DNA containing the LSU cistron (Whitfeld, personal communication).

These results imply that each of the RNA preparations contains nonribosomal chloroplast RNAs (messenger RNAs) that are complementary to the plastid DNA. Also, each RNA fraction contains discrete mRNA sequences; that is, the low molecular weight RNAs, 50-60 x 10^4 and 40-50 x 10^4 daltons, are not partially degraded 110-120 x 10^4 or 70-80 x 10^4 dalton messenger RNAs since they map to different regions of the chloroplast genome. Therefore, the chloroplasts of spinach contain at least four messenger RNA species transcribed from specific genes located on the chloroplast DNA molecules.

4.3. DISCUSSION OF CHLOROPLAST MESSENGER RNA PROPERTIES AND MAPPING

A study of chloroplast messenger RNA was initiated in the hope of obtaining pure messenger species, particularly for the LSU protein, and also some information about the chloroplast DNA coding functions. The principal difficulty in purifying chloroplast mRNAs has been due to their instability. This has prevented the use of multistep fractionation procedures such as have been successfully used elsewhere to purify non-polyadenylated mRNAs. The high sensitivity of chloroplast mRNAs to degradation is illustrated in Figure 4.28. Wheat leaf

Figure 4.28. Polysomes prepared from the second leaf of 10 day old wheat seedlings were incubated on ice in the presence or absence of ribonuclease as indicated. The profiles produced when the polysomes were separated on isokinetic sucrose gradients are shown with the size of the main polysome peaks indicated.

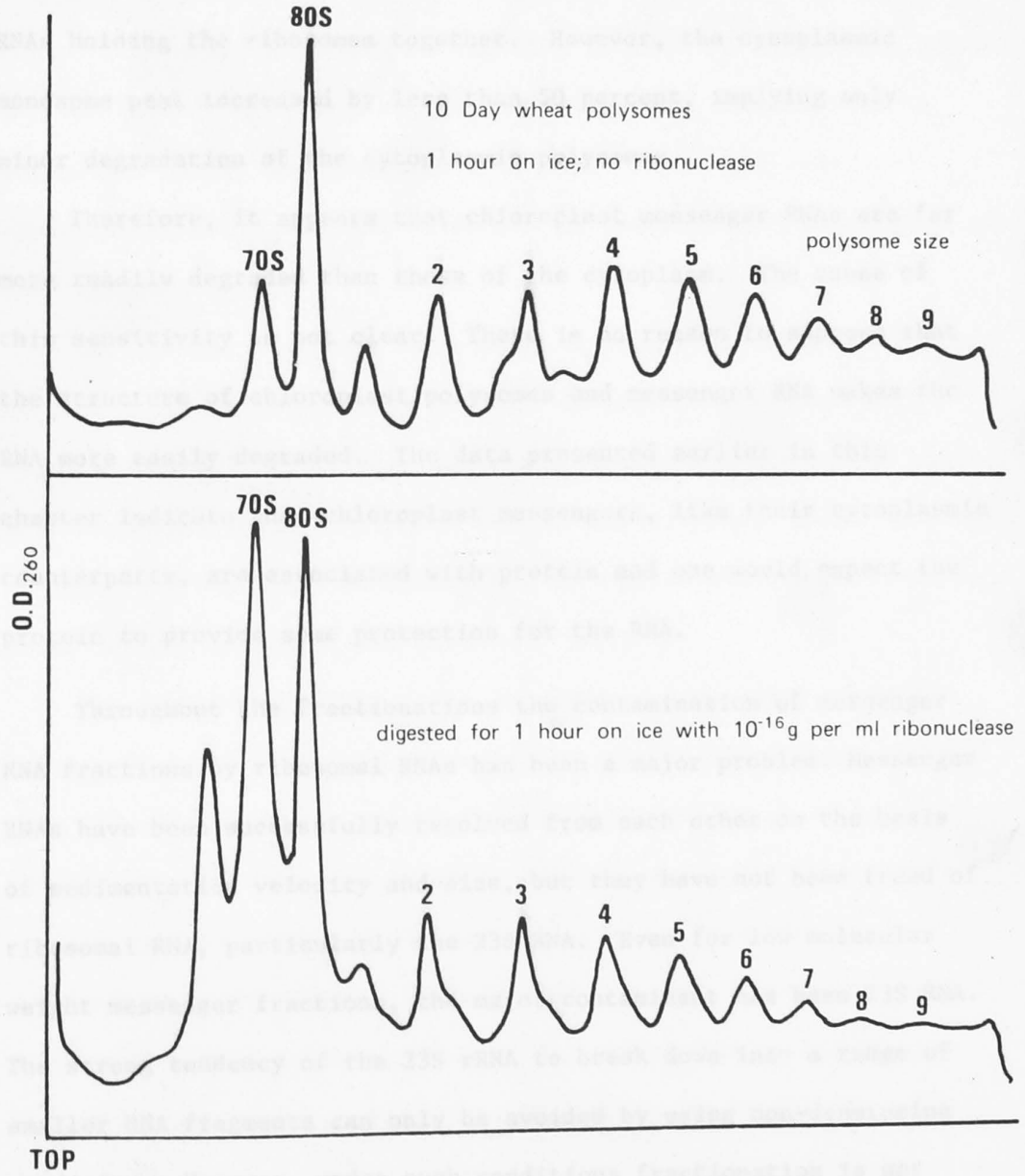


Figure 4.28. The digestion of total wheat leaf polysomes by pancreatic ribonuclease (sucrose gradient profiles)

polysomes were incubated in the presence of 10^{-16} g pancreatic ribonuclease per ml, on ice, for one hour. During this period there was a five-fold increase in the size of the chloroplast monosome peak, indicating extensive degradation of the messenger RNAs holding the ribosomes together. However, the cytoplasmic monosome peak increased by less than 50 percent, implying only minor degradation of the cytoplasmic polysomes.

Therefore, it appears that chloroplast messenger RNAs are far more readily degraded than those of the cytoplasm. The cause of this sensitivity is not clear. There is no reason to suppose that the structure of chloroplast polysomes and messenger RNA makes the RNA more easily degraded. The data presented earlier in this chapter indicate that chloroplast messengers, like their cytoplasmic counterparts, are associated with protein and one would expect the protein to provide some protection for the RNA.

Throughout the fractionations the contamination of messenger RNA fractions by ribosomal RNAs has been a major problem. Messenger RNAs have been successfully resolved from each other on the basis of sedimentation velocity and size, but they have not been freed of ribosomal RNA, particularly the 23S RNA. Even for low molecular weight messenger fractions, the major contaminant has been 23S RNA. The strong tendency of the 23S rRNA to break down into a range of smaller RNA fragments can only be avoided by using non-denaturing conditions. However, under such conditions fractionation is not efficient. It appears that the properties of spinach chloroplast RNAs are such that it will not be easy to obtain pure mRNA species by physical fractionation procedures.

The conformation and size of chloroplast messenger ribonucleoprotein particles of the spinach chloroplasts seem to be similar for all messenger species. The sedimentation velocity of these particles is between 20 and 30S but there is little fractionation of messengers within this range. The protein content of the RNAs is probably between 30 and 40 percent (based on buoyant density analysis). It appears that in the native state, the messenger RNAs exist in a tight spherical conformation similar to the ribosomes as assessed by sucrose gradient centrifugation.

Chloroplast messenger RNAs range in size from about 4×10^5 to over 11×10^5 daltons. They sediment in sucrose gradients at between 10 and 18S. In most cases the messenger RNA seems to have a hydrodynamic shape similar to that of the ribosomal RNAs. However, at least one mRNA species (the 1.1×10^6 dalton mRNA) appears to exist in solution as a long distended particle.

The large size of the 1.1×10^6 mRNA is surprising since its largest *in vitro* translation product is only 35,000 daltons (less than 300 amino-acids). In several RNA preparations which had been fractionated on urea-agarose gels, the high molecular weight mRNA species (greater than 1×10^6 daltons) yielded a 26,000 dalton polypeptide as its largest translation product. Therefore, the 1.1×10^6 dalton mRNA may be polycistronic, coding for at least two discrete polypeptides, namely the 35,000 and/or the 26,000 dalton polypeptides.

The possible location of the gene for the 1.1×10^6 dalton mRNA has been indicated on Figure 4.29 together with the region of the chloroplast genome hybridised to by the other three mRNA fractions.

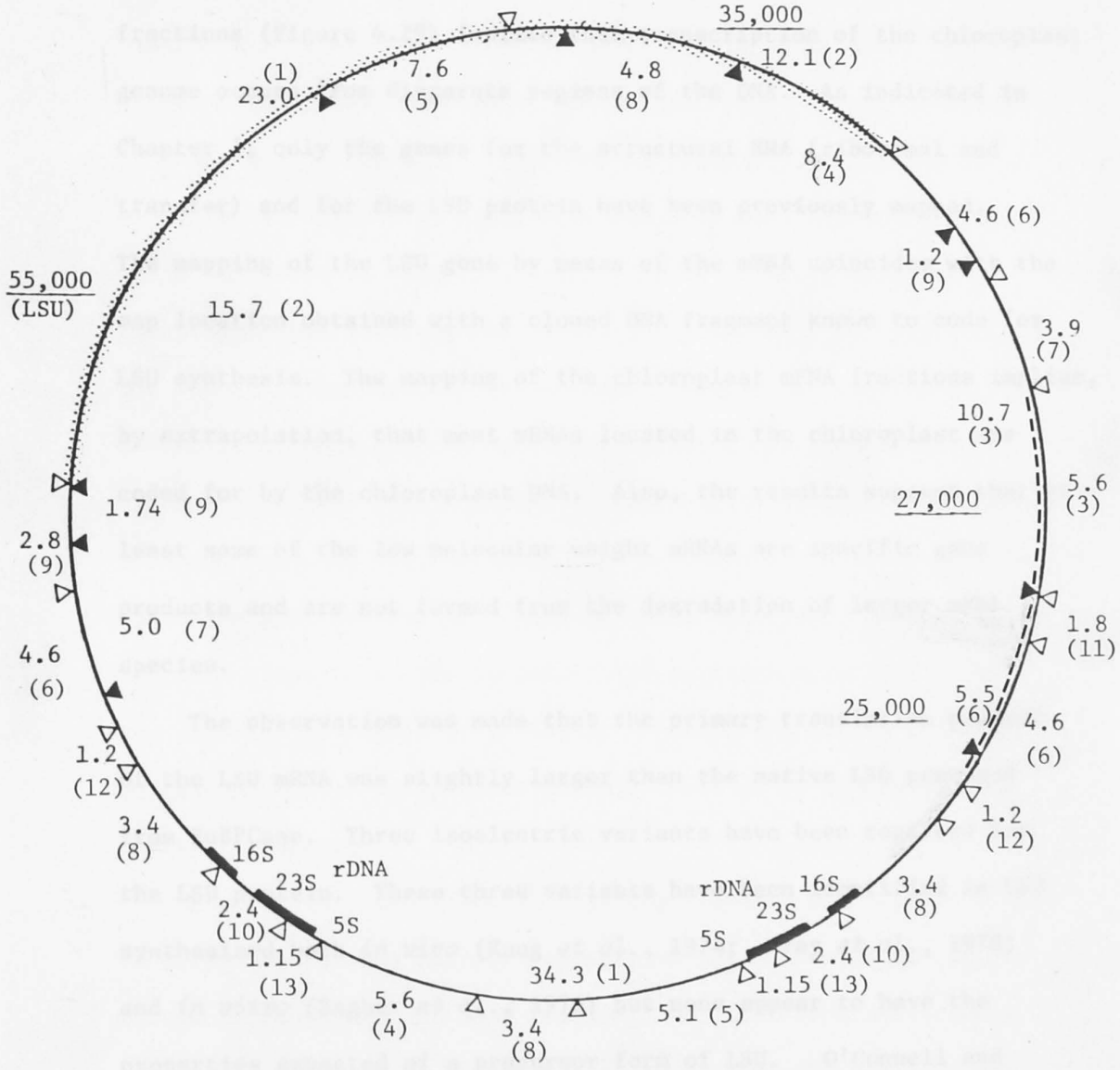


Figure 4.29. The location of four chloroplast mRNA fractions on restriction endonuclease maps of spinach chloroplast DNA (Pst and Sma maps compiled by Bohnert et al., 1978) Numbers underlined represent the size (in daltons) of the major protein synthesised *in vitro* by the mRNAs mapped. The DNA fragment sizes are also given (daltons x 10^6, not underlined) and the fragment number (in brackets).

In the absence of Bam and Hind III restriction endonuclease maps of spinach chloroplast DNA, a finer localisation has not been possible.

The wide spacing of the map locations of the four messenger fractions (Figure 4.29) implies that transcription of the chloroplast genome occurs from disparate regions of the DNA. As indicated in Chapter 1, only the genes for the structural RNA (ribosomal and transfer) and for the LSU protein have been previously mapped. The mapping of the LSU gene by means of the mRNA coincides with the map location obtained with a cloned DNA fragment known to code for LSU synthesis. The mapping of the chloroplast mRNA fractions implies, by extrapolation, that most mRNAs located in the chloroplast are coded for by the chloroplast DNA. Also, the results suggest that at least some of the low molecular weight mRNAs are specific gene products and are not formed from the degradation of larger mRNA species.

The observation was made that the primary translation product of the LSU mRNA was slightly larger than the native LSU prepared from RuBPCase. Three isoelectric variants have been reported for the LSU protein. These three variants have been identified in LSU synthesised both *in vivo* (Kung *et al.*, 1974; Gray *et al.*, 1978) and *in vitro* (Sagher *et al.*, 1976) but none appear to have the properties expected of a precursor form of LSU. O'Connell and Brady (1979) have suggested that these three variants are artifacts produced during the preparation of the proteins for isoelectric focussing. There has been no previous comment on an observed difference in apparent molecular weight of the initial and final LSU translation products. Perhaps gel fractionation procedures used here give higher resolution than has been achieved elsewhere.

It is surprising that LSU may be synthesised as a precursor, since it is made on soluble polysomes and exists in the cell as a soluble protein (RuBPCase). Most known cases of precursor proteins involve a polypeptide that is to be either inserted into, or be transported across, membranes. These precursors appear to function as a means of recognition for membrane transport mechanisms by providing a hydrophobic amino-acid chain (Blobel and Dobberstein, 1975; Dobberstein *et al.*, 1977). This is unlikely to be the function of the supposed LSU precursor since this polypeptide does not appear to be involved in membrane transport or insertion. Two alternative functions can be suggested for a LSU precursor.

LSU prepared from RuBPCase is essentially insoluble in aqueous solution below pH 9.3. However, the products of *in vitro* translation are soluble at neutral pH. This apparent difference in solubility may be a property of the secondary and tertiary structure of the two polypeptide chains or it may be the function of the precursor to render the LSU polypeptide soluble until it is assembled into the active enzyme.

The second possibility is that the proposed LSU precursor may be necessary for the correct assembly of the RuBPCase protein. Active RuBPCase has not been assembled *in vitro* from dissociated subunits. Also, it has recently been shown that the small subunit of RuBPCase is produced from its precursor in the stroma of the chloroplast and not in, or on, the membrane as the Blobell and Dobberstein model might have suggested (Smith and Ellis, 1979). It is still not known whether the processing of the SSU occurs before or after assembly of RuBPCase. Perhaps both LSU and SSU precursors are necessary for assembly.

The nature of the primary translation product of LSU may provide an explanation for the difficulties encountered here with the immunoprecipitation of *in vitro* synthesised LSU and of LSU polysomes. It is possible that the antibody preparations were unable to recognise the precursor LSU due to conformational differences between it and the processed polypeptide. This interpretation implies that the antibody preparations reacted with a different antigenic site as compared to the antibodies used by Gelvin and Howell (1977) and Sano *et al.*, (1979) to immunoprecipitate LSU polysomes.

A detailed analysis of the function and mechanisms of LSU and SSU processing and assembly may provide valuable information about the regulation of chloroplast gene expression and its interaction with the nuclear genome. The complexity of RuBPCase synthesis may be a feature of the synthesis of other chloroplast proteins, such as the coupling factor, where some subunits are nuclear-coded and others may be chloroplast-coded.

CHAPTER 5

Concluding Remarks

There were two principal aims in this project; to investigate the regulation of protein synthesis in plastids during the formation of chloroplasts from etioplasts and to obtain some information about the properties and gene locations of some chloroplast mRNAs. Plastid mRNAs provide the central theme since the transcription of the chloroplast DNA to produce mRNAs and the activity of the mRNAs as templates for translation provide the main sites for regulating the rate of protein synthesis.

Both quantitative and qualitative changes were shown to occur in plastid proteins during chloroplast formation. These changes were observed in the proteins accumulated in the plastids, in the proteins synthesised by isolated plastids and in the proteins made *in vitro* by plastid polysomes. Many of the protein changes were due to the production of nuclear-coded and cytoplasmically synthesised plastid proteins, such as the small subunit of RuBPCase. It has been demonstrated elsewhere (see Chapter 1) that both the transcription and translation of nuclear coded proteins is stimulated during greening and that the regulation of protein synthesis occurs at both the transcriptional and translational levels. However, neither the quantitative nor the qualitative changes in plastid proteins could be accounted for by variations in plastid mRNA levels or diversity when assessed by the *in vitro* translation of pea plastid RNA. There was a decline in mRNA levels per plastid during greening although the rate of protein synthesis increased. It was concluded, therefore, that transcriptional control was not responsible for the observed changes in plastid proteins during

greening. Although both mRNA and rRNA levels were shown to increase per apex during the etioplast to chloroplast transformation, RNA levels per plastid declined due to plastid division. The initial transcriptional response to light appeared to be an increase in the rate of rRNA synthesis. The synthesis of mRNAs began to accelerate later in the transformation. The apparent similarity of the *in vitro* translation products of etioplast and chloroplast RNAs suggested that the transcription of the genes coding for these mRNAs occurred at a constant relative rate during development of the chloroplasts; when the transcription of one gene increased, that of other genes showed a proportionate rise. The induction of transcription during greening may be due to a simple mechanism, such as a change in the type or activity of the chloroplast RNA polymerase(s?). The initial transcription of ribosomal genes may require one type of polymerase and the transcription of the mRNA genes an alternative or modified polymerase.

The assessment of changes in the level and type of plastid mRNAs during greening is based upon the assumption that the *E. coli* cell-free translation system is giving a reliable estimation of the diversity and abundance of messenger RNAs. Four distinct mRNA size classes from spinach chloroplasts were examined and found to have different *in vitro* translation products and different locations on the plastid genome. These observations supported the assumption that *in vitro* translation of plastid RNA extracts indicated the level and diversity of mRNAs in the extract.

Since the observed changes in plastid proteins during greening did not seem to be due to control of the transcription of the plastid genome, it was concluded that control operated on the translation of plastid mRNAs. The control of protein synthesis, by altering the rate at which mRNAs are translated, can be exerted in a number of ways. During pea chloroplast development two mechanisms appear to operate. Firstly, it was observed that the synthesis of plastid membrane proteins responded more slowly to the transfer of etiolated plants to the light than did soluble proteins, although the synthesis of membrane proteins was more strongly affected by greening. It was found that the proportion of ribosomes associated with membranes changed during development in accordance with changes in the synthesis of membrane proteins. This resulted in the suggestion that the observed delay in membrane protein synthesis was due to the time required for the formation of membranes and the assembly of active polysomes on the growing chloroplast membrane system. This apparently simple control system may account for the differential synthesis of plastid membrane proteins compared to soluble proteins.

The second mechanism of translational control that appears to operate during chloroplast formation lies in the ability of plastid mRNAs to compete differentially for ribosomes. The time required for ribosomes to bind to the mRNAs and for protein synthesis to begin, was found to vary during development (see Chapter 3). This may be the major mechanism for controlling the synthesis of specific proteins. In the case of one plastid protein, the LSU of RuBPCase, translational control appeared to completely account for changes in LSU synthesis relative to other plastid proteins during greening. Translational control seemed to operate only at the

initiation of LSU synthesis. There was no evidence to suggest that regulation of LSU chain elongation or termination occurred.

The translational control of plastid protein synthesis may be a function of the properties of the mRNA molecules themselves. No evidence was obtained that demonstrated a correlation between mRNA structure and template activity of the mRNAs. However, one chloroplast mRNA species was shown to have a hydrodynamic shape quite unlike that of most chloroplast mRNAs. This suggests that variation does exist in the shape and conformation of chloroplast mRNAs. Proteins associated with mRNAs may also affect the template activity of mRNAs. Chloroplast mRNAs were observed by buoyant density and sucrose gradient fractionation to be associated with protein in ribonucleoprotein particles. Although far from being strongly established, a high molecular weight messenger RNA species was identified in spinach chloroplast that may be polycistronic; two possible polypeptide products of this mRNA were detected. Polycistronic mRNAs are unusual in eukaryote cells and their presence may indicate specialised and specific control mechanisms.

Chloroplast mRNAs were found to be highly unstable. However, the control of protein synthesis in the plastids, via mRNA degradation and turnover, does not appear to be significant during greening since, as already discussed, no changes in the abundance of particular mRNA species, relative to others, were observed. The degradation of mRNAs only seems to affect protein synthesis late in development when protein synthesis in the chloroplasts is slowing down. No specificity for particular mRNA species was observed during this degradative phase, although rRNAs appeared to be less susceptible than mRNAs.

Post-translational modification of plastid-synthesised proteins may provide a further site for regulating the production and assembly of the final protein product. Grebanier *et al.*, (1979) noted that a 32,000 dalton protein was synthesised by chloroplasts as a 35,000 dalton precursor and subsequently cleaved to its final size and inserted into the chloroplast thylakoids. Some evidence has been presented in Chapter 4 to suggest that the LSU of RuPBCase may also be synthesised in precursor form. It is possible that the formation of RuBPCase is regulated at the stage of assembly of the oligomer by processing of the LSU and SSU (see Chapter 1) to their final sizes. The extent of post-translational modification and the significance of its role in the synthesis of proteins of chloroplasts is not known.

The results of experiments described in this thesis suggest that the control of plastid protein synthesis during the etioplast to chloroplast transformation, occurs primarily through the translation of plastid mRNAs. More specifically, the plastid mRNAs appear to vary in their ability to compete for ribosomes and initiate protein synthesis. This variation has been detected for a specific mRNA during development and also between mRNA species. The universality, stringency and actual mechanism of this control is not known, nor is it known whether this control operates during the differentiation and de-differentiation of other plastid types, such as amyloplasts, chromoplasts and proplastids.

APPENDIX I.

The Mathematics of Isokinetic Sucrose Density Gradients

Conventional linear sucrose gradient procedures suffer from two problems. Firstly, the determination of sedimentation constants is complicated. The molecules being sedimented slow down as they move away from the top layer due to the viscous drag of the medium increasing more rapidly than the centrifugal force. The viscosity and density of the medium changes according to complex equations along the sedimentation path, so that the mathematical computation of sedimentation velocities becomes too cumbersome for routine analysis. The use of tables prepared by computers can overcome this problem to some extent (McEwen, 1967). However, the gradients must be standardised carefully, since type of rotor, gradient, particle density, length and speed of centrifugation will all cause changes in the sedimentation.

In addition to causing these analytical and computational difficulties, the decrease in rates of sedimentation observed in linear gradients, seriously limits the resolving power of the method. The particles tend to pile-up towards the bottom of the tube. In most gradients the separation between two components fails to improve with sedimentation beyond a third to a half the length of the tube. Consequently, the potential resolution offered by the available length of path remains largely unexploited (Noll, 1967).

Both of these problems are overcome by constructing gradients in which "the linear increase in the driving force acting on a particle with increasing distance from the center of the rotor may be exactly compensated by an equivalent increase in the opposing forces of viscous drag and buoyancy" (McCarthy *et al.*, 1968). Isokinetic gradients have this property.

The equation describing the sedimentation coefficient of a particle in sucrose is given by the relationship:

$$S_{TM} = \frac{dx/dt}{\omega^2 x}$$

where: ω = angular velocity
x = distance moved
t = time
T = temperature
M = medium

in the standard state:

$$S_{20w} = S_{TM} \cdot \frac{\eta_{TM}(\rho_p - \rho_{20,w})}{\eta_{20,w}(\rho_p - \rho_{TM})}$$

$$\frac{dx}{dt} = S_{20w} \cdot \omega^2 x \frac{\eta_{20,w}(\rho_p - \rho_{TM})}{\eta_{TM}(\rho_p - \rho_{20,w})} \qquad (1)$$

Where: ρ_{TM} = density of sucrose solution

η_{TM} = viscosity of sucrose solution

p = particle

$\rho_{20,w}$ = density of water at 20°C (standard rate)

$\eta_{20,w}$ = viscosity of water at 20°C (standard rate)

ρ_p = particle density

Although ρ_{TM} and η_{TM} will vary down the centrifuge tube, the term $\frac{\eta_{20,w}}{\rho_p - \rho_{20,w}}$ will be constant. Therefore, let $a = \frac{\eta_{20,w}}{\rho_p - \rho_{20,w}}$

since $\eta_{20,w} = 1$ and $\rho_{20,w} = 1$, then $a = \frac{1}{\rho_p - 1}$.

We also know that ρ_{TM} and η_{TM} are both functions of the sucrose concentration. Therefore, let $\rho_{TM} = f(c)$ and $\eta_{TM} = g(c)$. Equation (1) can be written:

$$\frac{dx}{dt} = S_{20,w} \omega^2 x a \frac{(\rho_p - f(c))}{g(c)} \qquad (2)$$

In an isokinetic gradient $\frac{dx}{dt}$ (the rate of movement of the particle) must be constant. "$S_{20,w}$", "ω^2" and "a" are all constants, therefore the term $\frac{x(\rho_p - f(c))}{g(c)}$ must also be constant.

Let $\frac{x(\rho_p - f(c))}{g(c)} = K$ (3)

then (2) becomes $\frac{dx}{dt} = S_{20,w}\,\omega^2 aK$ (4)

Calculation of K

At the top of the centrifuge tube the viscosity and density of the sucrose solution will equal the viscosity and density of the lowest sucrose concentration used in the gradient. Therefore, $\eta(x) = \eta(x°)$ and $\rho(x) = \rho(x°)$ where x = distance from center of rotation and x° = top of the centrifuge tube.

Therefore, $$K = \frac{x°(\rho_p - \rho(x°))}{\eta(x°)} \quad (5)$$

since, x° is known (distance of the top of the centrifuge tube from the centre of rotation).

ρ_p is known (1.41 g/cm^3 for polysomes, 1.9 g/cm^3 for RNA, etc.).

ρ_o and η_o can be found from standard tables of density and viscosity of sucrose solutions,

then "K" can be calculated.

Placing the value for "K" in equation (3) it is now possible to vary "x".

$$\frac{x(\rho_p - f(c))}{g(c)} = K$$

therefore,

$$\frac{K}{x}(g(c)) + f(c) = \rho_p \quad (6)$$

Using tables of sucrose viscosity and density at various concentrations, we can determine "c" for varying "x" such that the relationship in equation (6) holds true. Two such values must be determined to allow subsequent calculations.

The shape of an isokinetic gradient will closely approximate to an exponential curve of sucrose concentration against distance down the tube (Noll, 1967). The equation of the gradient in the tube can be written:

$$c_x = c_i\, e^{-x/v} + c_r\,(1-e^{-x/v}) \qquad (7)$$

where: c_x = concentration of sucrose after "x mls" have been delivered.

c_i = initial sucrose concentration

c_r = concentration of reservoir sucrose

v = volume of mixing vessel

x = total volume delivered.

These terms are more easily understood if we examine the device for construction of isokinetic gradients. This design, shown in Figure A.1, is a modified form of the constant-volume device of Henderson (1969).

The equations presented have been derived by McCarty *et al.*, (1968). These authors proceeded to solve the equations for "v" and "c_r" using computer based calculations. However, it is possible to estimate "c_r" and "v" from equation (7) by means of values determined via equation (6) for "x" and "c".

Equation (7) states:

$$c_x = c_i e^{-x/v} + c_r(1-e^{-x/v})$$

therefore,

$$c_a = c_i c^{-x_a/v} + c_r(1-e^{-x_a/v})$$

and

$$c_b = c_i c^{-x_b/v} + c_r(1-e^{-x_b/v})$$

where c_i = initial sucrose concentration (arbitrary)

c_a and x_a) derived from equation (6), as
c_v and x_b) described earlier.

solving for "v":

$$v = -\frac{x_b}{\ln(\frac{c_b - c_r}{c_i - c_r})} \quad \text{and} \quad v = -\frac{x_a}{\ln(\frac{c_a - c_r}{c_i - c_r})} \qquad (8)$$

therefore;

$$(\frac{c_b - c_r}{c_i - c_r})^{x_a} - (\frac{c_a - c_r}{c_i - c_r})^{x_b} = 0 \qquad (9)$$

since c_a, c_b, c_i, x_a and x_b are known; c_r can be easily estimated.

Once c_r is known, "v" can be calculated from equation (8). The resulting gradient will be specific for centrifuge speed and rotor, and for the density of the particle being centrifuged.

Sample calculation

Gradient for separating RNA in an SW 41 rotor at 41,000 rpm;

temperature = 5°C.

Set initial sucrose concentration at 10 percent;

therefore, $c_i = 10$; $\eta_o = 2.19$; $\rho_o = 1.0400$ (from tables of density and viscosity of sucrose at 5°C).

The particle density of RNA is approximately 1.9 g/cm^3.

therefore;

$$K = \frac{x(\rho_p - \rho(x°))}{\eta(x°)} = 2.46 \qquad \text{(equation (5))}.$$

and

$$a = \frac{1}{\rho_p - 1} = 1.11$$

For SW 41 rotor $x_o = 6.8$ cm

$x_{max} = 15.23$ cm (maximum distance from centre of rotation)

since, $g(c) + \frac{x}{K} f(c) = \frac{x}{K} \rho_p$ (equation (6))

at 9 cm from the centre of rotation;

$$g(c) + 3.66\ f(c) = 6.95$$

The sucrose concentration must be 18.4 percent for these conditions to hold.

At 15 cm from the centre of rotation (near the bottom of the rate),

$$g(c) + 6.10\ f(c) = 11.59.$$

This corresponds to a sucrose concentration of 27.7 percent. Three data points are now known for the gradient (Table A.1).

Distance from centre of rotation (cm)	Sucrose concentration (percent)
6.8	10.0
9.0	18.4
15.0	27.7

Table A.1. Concentration of sucrose down an SW 41 centrifuge tube for an isokinetic gradient (5°C, RNA).

The values in Table A.1 can be used in equations (9) and, subsequently (7), to find "c_r" and "v".

c_r = 30.5 percent

v = 5.89 ml

The resulting gradient can be defined by the parameters:

c_i = 10 percent

c_r = 30.5 percent

v = 5.89 ml (from equation (7))

The time required for centrifugation of particular RNA species can be estimated from equation (2) if the sedimentation velocity of the particles is known.

$$\frac{dx}{dt} = S_{20,w}\,\omega^2 x a\left(\frac{\rho_p - f(c)}{g(c)}\right)$$

therefore:

$$S_{20,w} = \frac{x - x_o}{t\omega^2(aK)} \quad \text{and} \quad t = \frac{x - x_o}{S_{20,w}\omega^2(aK)} \qquad (10)$$

This gives "t" in seconds for known $S_{20,w}$.

For example, on the gradient calculated above, the 23S rRNA would move to 14.5 cm from the centre of rotation after 6.65×10^4 seconds (18½ hours) at 41,000 rpm (5°C).

The improved resolution of isokinetic gradients is shown in Figure A.2. Both gradients were run at 5°C, 41,000 rpm in the SW 41.

The isokinetic gradient has given 50 percent greater separation of 16 and 23S rRNA compared to the linear gradient (10 to 35 percent sucrose). The improved separation by isokinetic over linear sucrose gradients is consistently around 50 percent. The isokinetic gradients are easy to make, highly reproducible, and allow the rapid calculation of sedimentation velocities (from equation (10)).

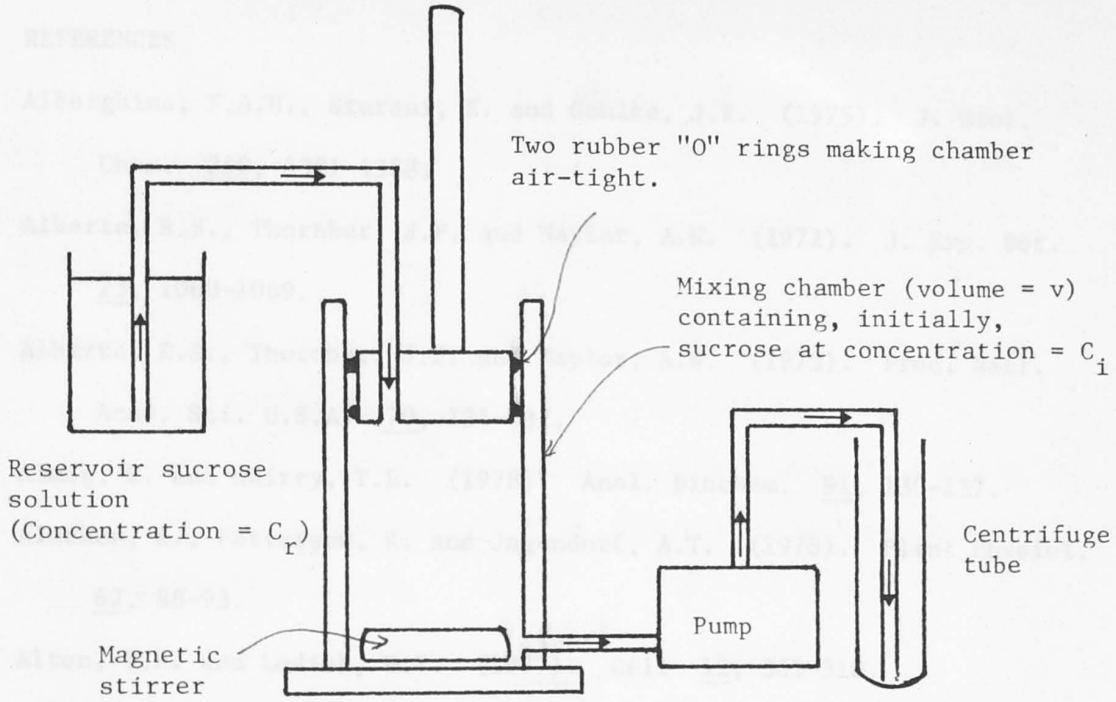


Figure A.1. Constant volume device for forming isokinetic sucrose gradients.

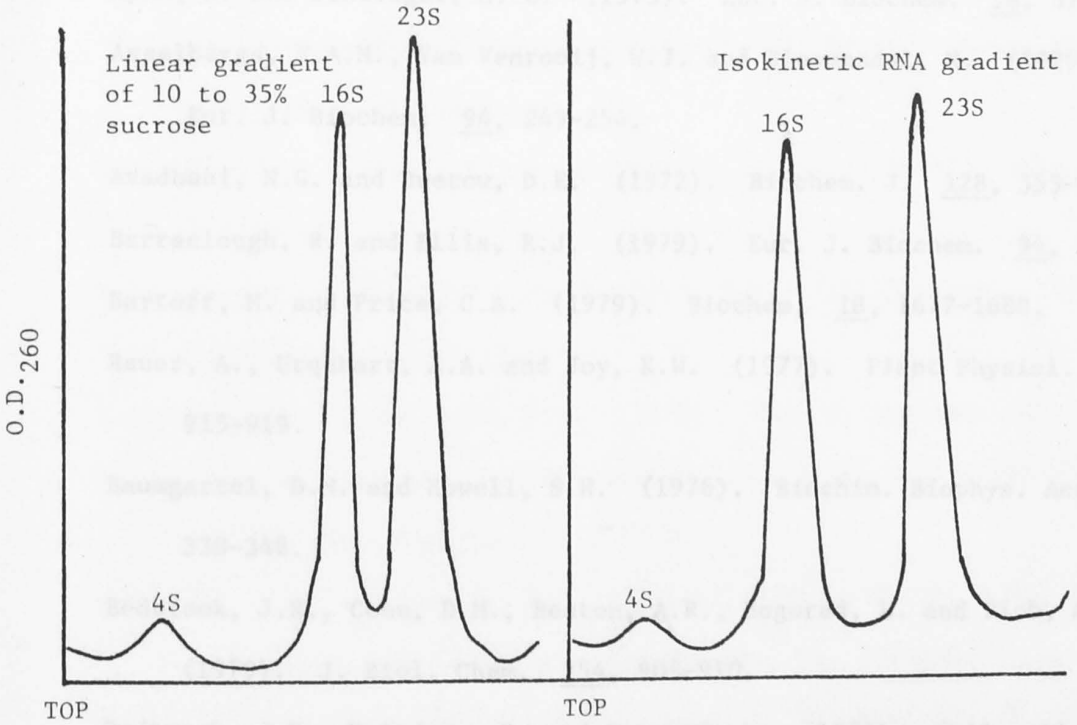


Figure A.2. Comparison of linear and isokinetic sucrose gradient separation of chloroplast RNA.

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